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EXHIBIT I

THE TRANSFORMING GROWTH FACTOR-β FAMILY

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INTRODUCTION

Secretory polypeptides are broadly used as mediators of intercellular communication to guide tissue development in metazoa. A decade ago, as the characterization of various mitogenic polypeptides and the isolation of scores of new ones were in progress, searches were launched to identify novel factors with activities other than promotion of cell growth. Some of the fruitful searches led to the isolation of a promoter of a transformed phenotype in fibroblasts, an inhibitor of cell proliferation, an inducer of chondrogenic differentiation, an inhibitor of myogenic differentiation, and an immunosuppressor. It was a major surprise to find that a single factor was responsible for all of these activities. Transforming growth factor type-β, or TGF-β, became the conventional name for this multifunctional factor, even though this name misrepresents the fact that TGF-β does not cause oncogenic transformation.

Besides being multifunctional, TGF-β represents a large family of factors with diverse activities. The concept that TGF-β is prototypic of a superfamily of growth, differentiation, and morphogenesis factors became clear in 1987 (Massagué 1987; Sporn et al 1987) following the rich harvest that yielded the inhibins, activins, Müllerian inhibiting substance, decapentaplegic product, and TGF-β2. One after another, these factors proved to be structurally related to TGF-β. This family now includes embryogenic morphogens, regulators of endocrine function, and broad-spectrum as well as specialized regulators of cell proliferation and differentiation. The distribution of TGF-β-related factors is widespread in organisms from fruit flies to humans, and their evolutionary conservation is unusually strict. These factors appear to be involved in many processes of tissue development and repair.

We have learned much about the structure, expression, and activity of the TGF-β-related factors, and their implications in physiology, pathology, and therapeutics. Some glimpses of their receptors and mechanisms of action have been caught too. Herein I will attempt to appraise the current status of the studies of the TGF-β family and point out some directions, challenges, and opportunities for the future.

THE TGF-β SUPERFAMILY

Prototype Structure

The structural prototype for this gene superfamily is the protein that was first isolated from human platelets as TGF-β (Assoian et al 1983), cloned from a human cDNA library (Derynck et al 1985), and later named TGF-

β1 (Cheifetz et al 1987). TGF-β1 is a disulfide-linked dimer of two identical chains of 112 amino acids. Each chain is synthesized as the C-terminal domain of a 390 amino acid precursor that has the characteristics of a secretory polypeptide; it contains a hydrophobic signal sequence for translocation across the endoplasmic reticulum and is glycosylated (Derynck et al 1985; Purchio et al 1988) (Figure 1). The precursor cleavage site is a sequence of four basic amino acids immediately preceding the bioactive domain.

This precursor structure is shared by all known members of the superfamily with the exception of the TGF-β4 precursor, which lacks a dis-

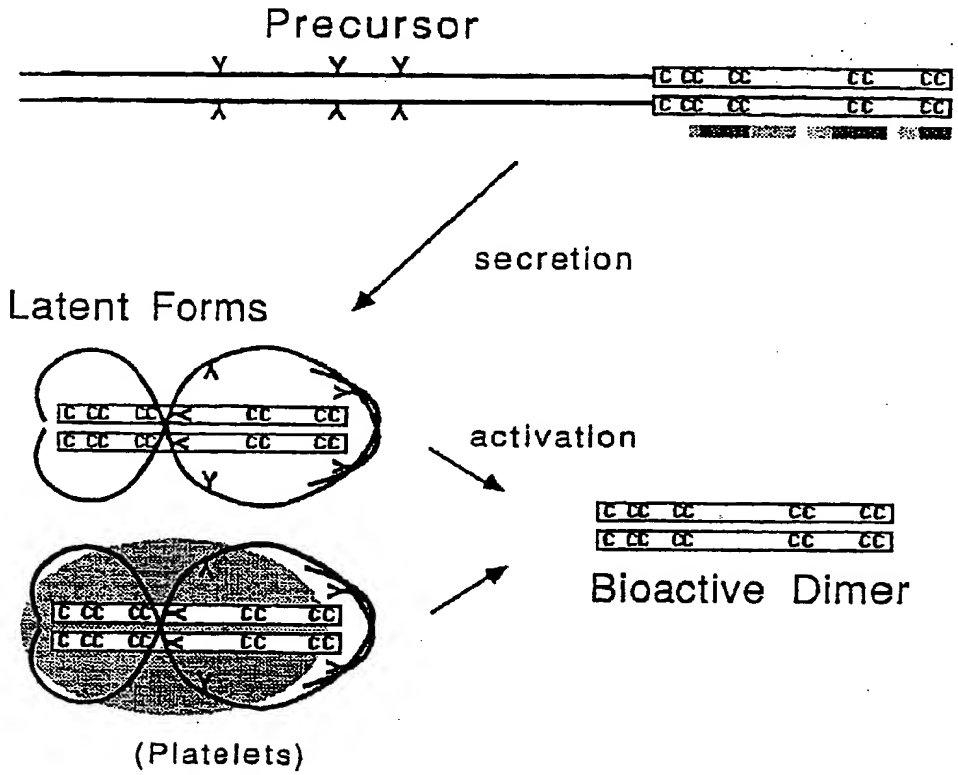

Figure 1 Precursor, latent, and bioactive forms of TGF-β1. The TGF-β1 precursor consists of an N-terminal signal sequence (*thin line*), a pro-region (*thick line*) and the C-terminal bioactive domain (*box*). The approximate locations of the three N-linked glycosylation sites (*Y*) in the pro-region and the 9 cysteines (*C*) in the bioactive domain are indicated. The intensity of the shadowing underlining the bioactive domain indicates the degree of amino acid sequence conservation throughout this domain in the other members of the TGF-β superfamily. After secretion, the cleaved pro-region remains associated with the TGF-β1 dimer forming a biologically latent complex. In platelets and certain cell lines, the latent complex also contains a 125–190-kd glycoprotein of unknown function (*shadowed*). Bioactive TGF-β1 is released by disassembly of this complex.

cernable signal sequence (Jakowlew et al 1988b). The known TGF-β-related factors can be grouped in four families according to their degree of structural or functional relationship (Table 1). Most of the amino acid sequence similarity between family members is in the C-terminal domain of the precursor. The degree of amino acid sequence identity in this domain ranges from 25 to 90% between different family members. The regions with highest homology are indicated in Figure 1. At least seven of the cysteines in this domain are conserved in all members of the superfamily, and all nine cysteines are conserved in the TGFs-β and the inhibin β chains. Except in Müllerian inhibiting substance (MIS) (Cate et al 1986), this domain is cleaved to generate a mature polypeptide similar in size to

Table 1 The TGF-β gene superfamily

	Chromosome		Bioactive dimers	
Gene	human	mouse	name	composition
TGF-β Family				
TGF-β1	19q13	7	TGF-β1	homodimer
TGF-β2	1q41	1	TGF-β2	homodimer
TGF-β3	14q24	2	TGF-β3	homodimer
TGF-β4[a]			(cDNA only)	
TGF-β5[b]			TGF-β5	homodimer
			TGF-β1.2	heterodimer
Inhibin Family				
α			Inhibin A	α.βA dimer
			Inhibin B	α,βB dimer
βA			Activin A	βA homodimer
βB				
			Activin AB	βA.βB dimer
DPP/VG1 Family				
DPP-C[c]			(cDNA only)	
Vg1[b]			(cDNA only)	
vgr-1			(cDNA only)	
BMP-2		13	BMP-2 homodimer	
BMP-3		2	homo or heterodimers	
BMP-4		5	(cDNA only)	
BMP-5		14,X	(cDNA only)	
BMP-6			homo or heterodimers	
BMP-7			homo or heterodimers	
Müllerian Inhibiting Substance Family				
MIS	19		MIS homodimer	

[a], [b], and [c] are from chick, *Xenopus*, and *Drosophila*, respectively. Chromosomal locations are from Dickinson et al (1990), Fujii et al (1986), Barton et al (1988) and ten Dijke et al (1988b).

mature TGF-β1. Most of the factors in the family have been isolated as dimers from natural or recombinant sources.

The N-terminal pro-region of a given factor may be conserved between animal species but is usually divergent between different factors (Derynck et al 1986; Cate et al 1986). Two potential functions of the pro-region are to assist in the folding of the bioactive domain during synthesis and, at least in the case of TGF-β1, to bind the mature factor forming a latent complex (see below).

TGFs-β: Forms, Expression, and Regulation

FORMS TGF-β is a term that refers to the dimeric products of various genes, five to date, identified by isolation of the proteins or by cDNA cloning. TGF-β was initially described as an activity produced by retrovirally-transformed cells (Roberts et al 1981), but it is now clear that TGFs-β are expressed in many normal cells and tissues and that this expression is not a unique attribute of the transformed phenotype. TGF-β1 has been purified from human and porcine blood platelets (Assoian et al 1983), which are the richest source of TGF-β1 (20 mg/kg), from human placenta (Frolik et al 1983), and bovine kidney (Roberts et al 1983).

Porcine platelets (Cheifetz et al 1987) and bovine bone (Seyedin et al 1987) yield TGF-β2 in addition to TGF-β1. TGF-β1 and 2 were identified in bone based on their cartilage-inducing activity, and they were named CIF-A and CIF-B before their identity with TGF-β1 and 2 was known (Seyedin et al 1985). TGF-β2 was also independently discovered by its activity as a growth inhibitor (Holley et al 1980) or as an immunosuppressor (Wrann et al 1987). TGF-β2 cDNAs have been cloned from human, monkey, and mouse libraries (de Martin et al 1987; Madisen et al 1988; Hanks et al 1988; Miller et al 1989a).

Human TGF-β3 was identified first at the cDNA level (ten Dijke et al 1988a; Derynck et al 1988) and was subsequently expressed in recombinant form (Graycar et al 1989; ten Dijke et al 1990). A chick embryo chondrocyte cDNA library yielded cDNAs corresponding to TGF-β3 and TGF-β4 (Jakowlew et al 1988a,b). TGF-β5 was identified as a cDNA from *Xenopus laevis* (Kondaiah et al 1990) and has been purified from *Xenopus* XTC cell cultures (Roberts et al 1989). Mammalian TGF-β4 and 5 have not been described yet. The complexity of this family may be greatly amplified by the existence of additional members and by the formation of heterodimers between different TGF-β gene products co-expressed in the same cell. The existence of the TGF-β1/TGF-β2 heterodimer (TGF-β1.2) has been confirmed in porcine platelets (Cheifetz et al 1987).

STRUCTURAL CONSERVATION The degree of identity between the five

mature TGF-β sequences ranges from 64% (TGF-β1 vs TGF-β4) to 82% (TGF-β2 vs TGF-β4) (Kondaiah et al 1990), but individually TGFs-β are extremely well conserved. Thus there is >97% identity between the mature TGF-β1 sequences from various mammalian and avian species (Derynck et al 1987; Jakowlew et al 1988c), and the same is true for TGF-β2 and 3 (Madisen et al 1988; Jakowlew et al 1988a). Conservation is also evident at the genomic level. The TGF-β1 gene in various mammalian species has a seven-exon structure (Derynck et al 1987; Van Obberghen-Schilling et al 1987) that is largely conserved in other TGF-β genes (Derynck et al 1988). This conservation suggests that the TGFs-β arose by duplication of a common ancestor. The various TGF-β genes, however, are located in separate chromosomes in both man and mouse (Table 1).

Multiplicity of TGF-β forms and sequence conservation within each form through evolution suggest important specific roles for each of the TGFs-β. Differences are manifested in the pattern of expression of the various TGFs-β in vivo (see below) and in their ability to interact with different cell surface receptors (Cheifetz et al 1987). Acting on cultured cells, TGF-β1, 2, and 3 often display similar activity and potency (Cheifetz et al 1987; Seyedin et al 1987; Graycar et al 1989), but show marked differences in certain cases (Ohta et al 1987; Ottmann & Pelus 1988; Tsunawaki et al 1988; Jennings et al 1988; Cheifetz et al 1990). Differences between the activity of TGF-β1 and 2 have also been noted in vivo (Rosa et al 1988). The high degree of conservation of the individual TGF-β sequences suggests the existence of evolutionary pressure to retain certain specific features of each of these factors. Such features should become apparent from a better characterization of their individual activities and the resolution of their three-dimensional structures.

EXPRESSION PATTERNS Numerous cell types in culture express one or multiple forms of TGF-β, at least at the mRNA level (Derynck et al 1988). In general, the pattern of expression of the different TGFs-β varies with each cell type and does not appear to be uniform among cells of the same lineage. Expression of TGF-β is active throughout embryonic development and into adulthood (Heine et al 1987; Rappolee et al 1988; Thompson et al 1989; Miller et al 1989a). Histochemical localization studies have shown expression of TGF-β1 and TGF-β2 mRNAs or proteins in discrete regions of many tissues with characteristic temporal patterns. In the mouse embryo, TGF-β1 mRNA is detectable in lung, intestine, and kidney mesenchymes, epithelial structures, megakaryocytes, osteocytes, and centers of hematopoiesis (Lehnert & Akhurst 1988; Wilcox & Derynck 1988). TGF-β2 mRNA is detectable in gastrointestinal and tracheal submucosae, blood vessels, skin, cartilage, and bone (Pelton et al 1989). TGF-β

immunostaining is high in mesodermal structures including teeth, larynx, palate, heart valves, cartilages, bones, and hair follicles (Heine et al 1987). At least 12 tissues and organs from adult mouse show expression of mRNAs for TGF-β1, TGF-β2, and/or TGF-β3 (Thompson et al 1989; Miller et al 1989a,b); TGF-β1 immunoreactivity is present in cells of the adrenal cortex, bone marrow, cardiac myocytes, chondrocytes, renal distal tubules, ovarian glandular cells, and chorionic cells of the placenta, and in extracellular matrices of the cartilage, skin, heart, pancreas, placenta, and uterus (Thompson et al 1989). The immunohistological distribution of a particular TGF-β form does not always match the distribution of the corresponding mRNA, a discrepancy that could result from diffusion and accumulation of the protein away from the sites of synthesis, a lack of translation of the mRNA (Assoian et al 1987), or immune cross-reactivity with other forms of TGF-β.

CONTROL OF ACTIVITY The existence of mechanisms that tightly control the expression and activity of TGF-β may be expected because numerous cell types express and can respond to these factors. TGF-β expression and activity are controlled by (*a*) regulation of TGF-β gene transcription, (*b*) production of TGF-β as a latent factor, and (*c*) sequestration of activated TGFs-β by extracellular matrix and circulating proteins.

Transcription of the TGF-β1 gene can be stimulated by phorbol esters presumably via a protein kinase C-dependent pathway (Akhurst et al 1988), and by TGF-β1 itself (Van Obberghen-Schilling et al 1988). The 5′ region of the TGF-β1 gene contains two transcription start sites (Kim et al 1989a); one promoter site located upstream of the first transcriptional start site and a second located between the two start sites (Kim et al 1989b), as well as several transcriptional inhibitory regions (Kim et al 1989a, 1990). Both promoters contain transcriptional enhancer elements that respond to induction by phorbol esters and TGF-β1, or transactivation by AP-1 (Kim et al 1990). Activation via these elements is mediated by binding of the (Jun-Fos) AP-1 complex (Kim et al 1990). Additional putative phorbol ester responsive elements are present in the 3′ flanking region of the TGF-β1 gene (Scotto et al 1990). Since expression and activity of *jun* and *fos* genes are modulated by numerous factors including their own products (Sassone-Corsi et al 1988; Schütte et al 1989) as well as TGF-β1 (Pertovaara et al 1989), these mechanisms have the capacity to finely tune TGF-β1 expression in response to diverse stimuli.

With the exception of platelets, where TGF-β is stored in α-granules (Assoian & Sporn 1986), the TGFs-β appear to be released from cells via a constitutive secretory pathway. TGF-β1, however, is released from either platelets or cultured cell lines as part of an inactive complex unable to

interact with cell surface receptors (Lawrence et al 1985; Pircher et al 1986). Exposure to extreme pH (<4 or >9), chaotropic agents (sodium dodecyl sulfate, urea) or plasmin in vitro release active TGF-β from the latent complex (Lawrence et al 1985; Lyons et al 1988). The latent complex isolated from human platelets and fibroblasts consists of the mature TGF-β1 dimer plus two TGF-β1 pro-region polypeptides disulfide-linked to a glycoprotein of 125–160 kd in platelets, or 170–190 kd in fibroblasts (Miyazono et al 1988; Wakefield et al 1988; Kanzaki et al 1990). The pro-region polypeptides are also disulfide-linked to each other. The amino acid sequence deduced from the 125–160 kd glycoprotein cDNA contains multiple EGF-like repeats in tandem as the main distinctive feature (Kanzaki et al 1990). The function of this protein is unknown at the moment. This protein does not prevent binding of activated TGF-β1 to cells, has no detectable proteolytic activity, and does not appear to bind activated TGF-β1, or to be related to the TGF-β-binding proteoglycan, betaglycan (see below).

Studies with cells that overexpress a transfected TGF-β1 gene, however, indicate that association of mature TGF-β1 with the pro-region is sufficient to retain this factor in the latent state (Gentry et al 1988). Glycosylation and dimerization of newly translated TGF-β1 precursor are followed by cleavage of the mature domain that continues to interact with the pro-region after release from the cell (Gentry et al 1988). The pro-region appears to be essential for the correct folding of TGF-β1 during synthesis (Gray & Mason 1990). The TGF-β1 pro-region contains mannose 6-phosphate (Purchio et al 1988) as well as the arg-gly-asp (RGD) sequence that in fibronectin, vitronectin, laminin, and other cell adhesion molecules recognizes certain adhesion receptors of the integrin class (Ruoslahti & Pierschbacher 1987). The TGF-β1 pro-region can bind to cell surface mannose 6-phosphate receptors (Kovacina et al 1989), but it is not known whether the RGD sequence can mediate binding of proTGF-βs to integrins, or whether binding mediated by RGD or mannose 6-phosphate can lead to activation of latent TGF-β1. The precise mechanisms that activate latent TGF-β in vivo are also unknown. Endothelial cell cultures can activate latent TGF-β, but only when cells are in contact with vascular pericytes (Antonelli-Olridge et al 1989; Sato & Rifkin 1989). Evidence suggests that the proteolytic action of plasmin or cathepsin D on the TGF-β1 pro-region (Lyons et al 1988; Sato & Rifkin 1989), the removal of carbohydrate residues in this region (Miyazono & Heldin 1989), and the action of acidic microenvironments in sites of wound healing and bone resorption might contribute to activate latent TGF-β1 in vivo.

Once released from the latent complex, active TGF-β1 can be bound by various extracellular matrix components and serum proteins. Clearance

of circulating activated TGF-β is extremely rapid (<3 min; Coffey et al 1987) and binding to α2-macroglobulin might be involved in this process (O'Connor-McCourt & Wakefield 1987). TGF-β can accumulate in interstitial matrices (Thompson et al 1989). High affinity binding of TGF-β to the core protein of the proteoglycan, betaglycan (Andres et al 1989), or lower affinity interactions with abundant matrix components, might protect TGF-β from degradation, or might function as a long-term reservoir, sustained release mechanism, or TGF-β clearance system.

Inhibins and Activins

Inhibins and activins are dimeric polypeptides initially isolated from ovarian follicular fluid based on their ability to modulate the production of follicle-stimulating hormone (FSH) from pituitary cells (Ling et al 1985). The inhibins are composed of an α subunit and either a βA subunit (inhibin A), or βB subunit (inhibin B) (Mason et al 1985; Forage et al 1986). These heterodimers inhibit production of pituitary FSH, gonadal sex steroids, and placental hormones (Mason et al 1985; Forage et al 1986; Ying et al 1986b; Petraglia et al 1989). The amino acid sequences of the two β subunits are 60% identical. The α subunit sequence is as divergent from the β subunits (~25% identity at the C-terminal domain) as it is from the other members of the TGF-β family. There is substantial evidence indicating that inhibins are indeed physiologic regulators of FSH production (Rivier et al 1986). Inhibins appear to repress FSH production by decreasing FSH-α and FSH-β mRNA levels (Attardi et al 1989).

In addition to dimerizing with the α subunit, βA and βB chains can pair with each other (Vale et al 1986; Ling et al 1986). Remarkably, the resulting dimers βA-βA and βA-βB have biological activity opposite to that of inhibins. These dimers, called activins, stimulate FSH production by pituitary cell cultures, steroidogenesis in granulosa cells, and production of gonadotropin-releasing hormone, chorionic gonadotropin, and progesterone in cultured human placenta cells (Vale et al 1986; Ling et al 1986; Petraglia et al 1989). Furthermore, some of these actions are mimicked by TGF-β1 (Ying et al 1986a). The actions of inhibins and activins are not restricted to gonadal and pituitary cells. Homodimeric βA activin was independently identified as a factor produced by human leukemia cells capable of inducing differentiation of erythroleukemia cells (Eto et al 1987). This action is antagonized by inhibins but, in contrast to other actions, it is not mimicked by TGF-β1 (Eto et al 1987; Yu et al 1987). More recently, expression of α, βA, and βB subunits has been detected in many other extragonadal tissues (Meunier et al 1988). Remarkably, activin A is produced by *Xenopus laevis* XTC cells in culture and has potent

mesoderm-inducing activity in mid-blastula *Xenopus* embryo explants (Smith et al 1990).

The antagonistic activity of inhibins and activins raises questions concerning their mode of action. Do these factors oppose each other by acting through receptors that produce signals of opposite sign, or do they compete antagonistically for the same receptors? Activins are antagonized not only by inhibins, but also by follistatin, an activin-binding protein found in follicular fluid and other tissues (see below). Thus, as in the case of TGF-β, complex mechanisms appear to ensure a tight control of the activity of these factors.

Decapentaplegic, Vg1, and BMPs

The biology of these TGF-β-related factors underscores the role of certain members of this family as morphogens in arthropod and vertebrate developmental processes. The decapentaplegic (DPP) gene complex encodes important functions in embryonic as well as larval *Drosophila* pattern formation. Mutations in various regions of the DPP gene complex result in failed dorsal-ventral patterning during early embryogenesis and defective patterning of the larval imaginal disks (Spencer et al 1982). The mutations affect *cis*-regulatory elements that control the expression of a set of overlapping transcripts. The product encoded by these transcripts has the predicted structure of a TGF-β-related molecule, with a C-terminal sequence that is 36% identical to the mature TGF-β1 sequence (Padgett et al 1987).

Another member of the family, Vg1, is involved in embryonic development in *Xenopus laevis*. Vg1 is encoded by a maternally inherited mRNA that is restricted to the vegetal (endodermal) pole of the embryo (Weeks & Melton 1987). The Vg1 product is $\sim$38 and $\sim$50% identical, respectively, to TGF-β1 and DPP. The presence of maternal Vg1 mRNA persists through the process of mesoderm induction, in which it may participate, and declines sharply after gastrulation (Melton 1987). Vgr-1, a mouse cDNA isolated by its homology to Vg1, encodes a product whose predicted C-terminal domain is roughly as similar ($\sim$60% identity) to the corresponding sequence in the Vg1 product as to the DPP product and to BMP-2 (Lyons et al 1989). Vgr-1 expression in the mouse increases throughout development and into adulthood in many tissues and is induced during endodermal differentiation of F9 teratocarcinoma cells.

Bone has a unique capacity for self-restoration. An activity extracted from bone, and designated bone morphogenetic protein (BMP), induces chemotactive, proliferative, and differentiative responses that culminate with the transient formation of cartilage followed by accumulation of bone

with hematopoietic marrow (Wozney 1989). This process is quite distinct from promotion of growth of preexisting bone, an action that can be induced by various growth factors and hormones including TGF-β1. Purified BMP preparations contain multiple component polypeptides (Wang et al 1988). N-terminal sequencing and molecular cloning of these components has shown that with the exception of BMP-1, the BMPs are members of the TGF-β superfamily and are particularly similar to DPP and Vg1 (Wozney et al 1988; Wozney 1989). The predicted amino acid sequences of human and bovine BMP-2 (formerly BMP-2A) and BMP-4 (formerly BMP-2B) show the highest degree of C-terminal domain identity (~75%) with *Drosophila* DPP, and they may represent the mammalian counterparts of this protein. BMP-5, 6, and 7 are closely related to each other and, like BMP-3 (also named osteogenin, Luyten et al 1989), show ~60% identity in their C-terminal domain with BMP-2 (Wozney 1989). BMP-2, 3, 6, and 7 have been isolated from human and/or bovine bone and are bioactive as TGF-β-like homodimers or heterodimers. BMP-4 and 5 are known only at the cDNA level. Physiologically, BMPs may act in concert with other factors to induce properly balanced bone formation. Recombinant BMP-2 implanted alone, however, is sufficient to induce ectopic bone production (Wang et al 1990).

Müllerian Inhibiting Substance

Müllerian inhibiting substance (MIS), also known as anti-Müllerian hormone, was identified and later purified based on its ability to induce regression of the primordium of female genitalia, the Müllerian duct, in mammalian male embryos (Blanchard & Josso 1974). MIS is produced by Sertoli cells of the fetal and adult testis and by ovarian granulosa cells after birth (Blanchard & Josso 1974; Vigier et al 1984). The deduced sequence of MIS C-terminal domain is ~25% identical to that of the other TGF-β-related factors. Purified from testes, MIS is a disulfide-linked homodimer of 70–74-kd glycosylated chains that, in contrast to the other TGF-β-related factors, contains the glycosylated N-terminal extension uncleaved from the C-terminal domain (Cate et al 1986). Recombinant MIS expressed in cultured cells, however, can undergo TGF-β-like proteolytic processing (Pepinski et al 1988). It is conceivable that the bioactive MIS entity could be generated by cleavage of the precursor at the sites of action in vivo.

In addition to its activity as a regulator of gonadal morphogenesis, MIS can act on ovarian endocrine differentiation. In organ culture, MIS induces endocrine sex reversal in fetal ovaries with release of testosterone instead of estradiol (Vigier et al 1989). This action appears to result from decreased

aromatase activity caused by MIS and correlates with the formation of seminiferous cord-like structures. Expression of MIS led to abnormal sexual development including the progressive degeneration of the ovaries in transgenic female mice, and overexpression of MIS led, paradoxically, to feminization and regression of genitalia in transgenic male mice (Behringer et al 1990). These observations suggest that MIS may promote testicular morphogenesis in addition to Müllerian duct regression. As with other TGF-β-related factors, progress in MIS research is revealing a broader range of actions than was originally anticipated.

BIOLOGICAL ACTIONS OF TGF-β

Most of the current information on the activity of TGF-β derives from the study of TGF-β1, 2, and 3. As mentioned above, these TGF-β1 isoforms acting on cultured cells often display similar activity and potency, but occasionally show marked differences (Ohta et al 1987; Ottmann & Pelus 1988; Tsunawaki et al 1988; Jennings et al 1988; Cheifetz et al 1990).

The ability of TGF-β to elicit multiple cellular responses, including responses of opposite sign, has been a subject of great interest. The paradigm of TGF-β as a dual factor emanated first from studies on cell proliferation; depending on the conditions, TGF-β can either inhibit or stimulate proliferation (Tucker et al 1984; Moses et al 1985; Roberts et al 1985; Massagué et al 1985). In some instances, the mechanism that leads to this duality is apparent. For example, TGF-β1 action slows the cell cycle of AKR-2B mouse fibroblasts (Shipley et al 1985), but induces expression of platelet-derived growth factor-B (PDGF-B) in these cells (Leof et al 1986). Thus when AKR-2B cells are placed in a mitogen-rich medium, there is a net growth inhibitory effect of TGF-β1, but when AKR-2B cells are plated in a mitogen-free medium, there is a net growth stimulatory effect caused by TGF-β1-induced autocrine PDGF. In another example, TGF-β decreases the proliferation rate of NRK-49F rat fibroblasts cultured as monolayers in the presence of EGF (Roberts et al 1985), but allows these anchorage-dependent cells to grow in a semisolid medium by inducing the production of a fibronectin-collagen-proteoglycan extracellular matrix to which the cells can adhere (Ignotz & Massagué 1986; Bassols & Massagué 1988). Many of the diverse effects of TGF-β on cell proliferation and phenotype are less susceptible to simple explanations. As discussed below, important cell-specific determinants dictate the nature of a cell's response to TGF-β. Comprehensive accounts of the response of individual cell types to TGF-β are available (Roberts & Sporn 1990; Ignotz & Massagué 1990). The following summarizes some important aspects of the biological activity of the TGFs-β.

Control of Cell Proliferation

The concept that cell proliferation can be restrained not only by limitations in the supply of mitogenic stimuli, but also by the action of negative growth regulators (Holley et al 1980), has been substantiated by the identification of TGFs-β as some of the most potent growth inhibitors known to date (Tucker et al 1984). All TGF-β forms tested display reversible growth inhibitory activity in normal as well as transformed epithelial, endothelial, fibroblast, neuronal, lymphoid, and hematopoietic cell types (Tucker et al 1984; Moses et al 1985; Roberts et al 1985; Shipley et al 1986; Kehrl et al 1986a; Frater-Schröder et al 1986; Carr et al 1986; Cheifetz et al 1987, 1990; Knabbe et al 1987; Ohta et al 1987; Kimchi et al 1988; Graycar et al 1989). In certain cell lineages, TGF-β opposes the action of specific mitogens such as EGF in keratinocytes (Coffey et al 1988), IL-1 and IL-2 in lymphocytes (Ristow 1986; Kehrl et al 1986a,b; Wahl et al 1988), and IL-3, GM-CSF, and CSF-1 in hematopoietic progenitor cells at different stages of differentiation (Ohta et al 1987; Keller et al 1988). The extent of the growth inhibitory response to TGF-β varies with the cell type and reaches a virtual growth arrest in certain lung epithelial cells, lung fibroblasts and keratinocytes (Tucker et al 1984; Shipley et al 1986; Chambard & Pouyssegur 1988). TGF-β acts by lengthening or arresting the G1 phase of the cell cycle (Shipley et al 1985; Nakamura et al 1985; Heimark et al 1986; T. Lin et al 1987; Laiho et al 1990).

Evidence for an antiproliferative action of TGF-β1 in vivo has been obtained with inert polymer beads impregnated with TGF-β1 and implanted near the epithelial end buds of immature mammary glands (Silberstein & Daniel 1987). Also, intravenous injection of TGF-β1 or 2 has a negative effect on the proliferative response of regenerating rat liver (Russell et al 1988). TGF-β1 mRNA is expressed in terminally differentiating cells adjacent to the suprabasal layer of phorbol ester-treated epidermis, which suggests a role in epidermal cell withdrawal from the proliferative state (Akhurst et al 1988). TGF-β is expressed in apical cells from enteric villi but not in proliferative crypt enterocytes (Barnard et al 1989). The antiproliferative action of TGF-β on B-lymphocytes (Kehrl et al 1986a), T-lymphocytes (Kehrl et al 1986b), and thymocytes (Ristow 1986) is one of the components of the immunosuppressive activity that these factors display in vitro and in vivo (Wrann et al 1987; de Martin et al 1987) (see below).

TGF-β can also stimulate cell proliferation although, as mentioned above, the mitogenic effect may be secondary to other cellular responses. TGF-β stimulates proliferation of NRK-49F and AKR-2B fibroblasts plated in a semisolid medium (Roberts et al 1981; Moses et al 1981) or in

a mitogen-poor medium (Massagué 1984; Leof et al 1986), and of early human embryo fibroblasts (Hill et al 1986) and rat calvaria osteoblasts (Centrella et al 1987).

Control of Cell Adhesion

Cell migration, homing, and settlement during tissue formation, repair, tumor invasion, and metastasis are guided by a complex set of adhesive interactions between cells and extracellular matrices. Many of the cell surface components mediate adhesion of cells to the extracellular matrix and to other cells. The adhesive behavior of a cell is determined in part by the type and level of adhesion receptors that it expresses, and the type of extracellular matrix that it produces and with which it interfaces. In addition to providing physical support, adhesive interactions are a major conduit for intercellular regulation of cell function and phenotype. The possibility that the cell adhesion apparatus and the composition of extracellular matrices might be regulated by growth and differentiation factors is clearly apparent in the action of TGF-β on many cell types.

The action of TGF-β on normal mesenchymal, epithelial, and lymphoid cells, as well as various tumor cell lines, generally leads to up-regulation of cell adhesion. This action is mediated in concert by enhanced synthesis and deposition of extracellular matrix components, decreased pericellular proteolysis, and modification of the repertoire of cell surface adhesion receptors, as summarized in Figure 2. The original impetus to examine the expression of these molecules in response to TGF-β came from the observation of TGF-β effects on cell morphology and differentiation (Ignotz & Massagué 1985) and its involvement in wound healing, a process with intense accumulation and remodeling of the extracellular matrix (Roberts et al 1986). The marked and generalized effect of TGF-β on extracellular matrices is likely to play a major role in processes of organismal morphogenesis and development, tissue repair processes, and the pathogenesis of certain fibrotic diseases.

CONTROL OF EXTRACELLULAR MATRIX PROTEIN EXPRESSION TGF-β action elevates fibronectin expression in several mesenchymal and epithelial cell types, both normal and transformed (Ignotz & Massagué 1986; Like & Massagué 1986; Dean et al 1988). Up to tenfold elevation in fibronectin synthesis, and a corresponding increase in extracellular matrix fibronectin accumulation, are frequently observed in response to TGF-β1 (Ignotz et al 1987; Dean et al 1988). TGF-β also regulates the expression of type I collagen α1 and α2 chains, and collagen types III, VI, and X (Ignotz & Massagué 1986; Roberts et al 1986; Varga et al 1987). Expression of type II collagen is induced in mesenchymal muscle cells secondarily to their

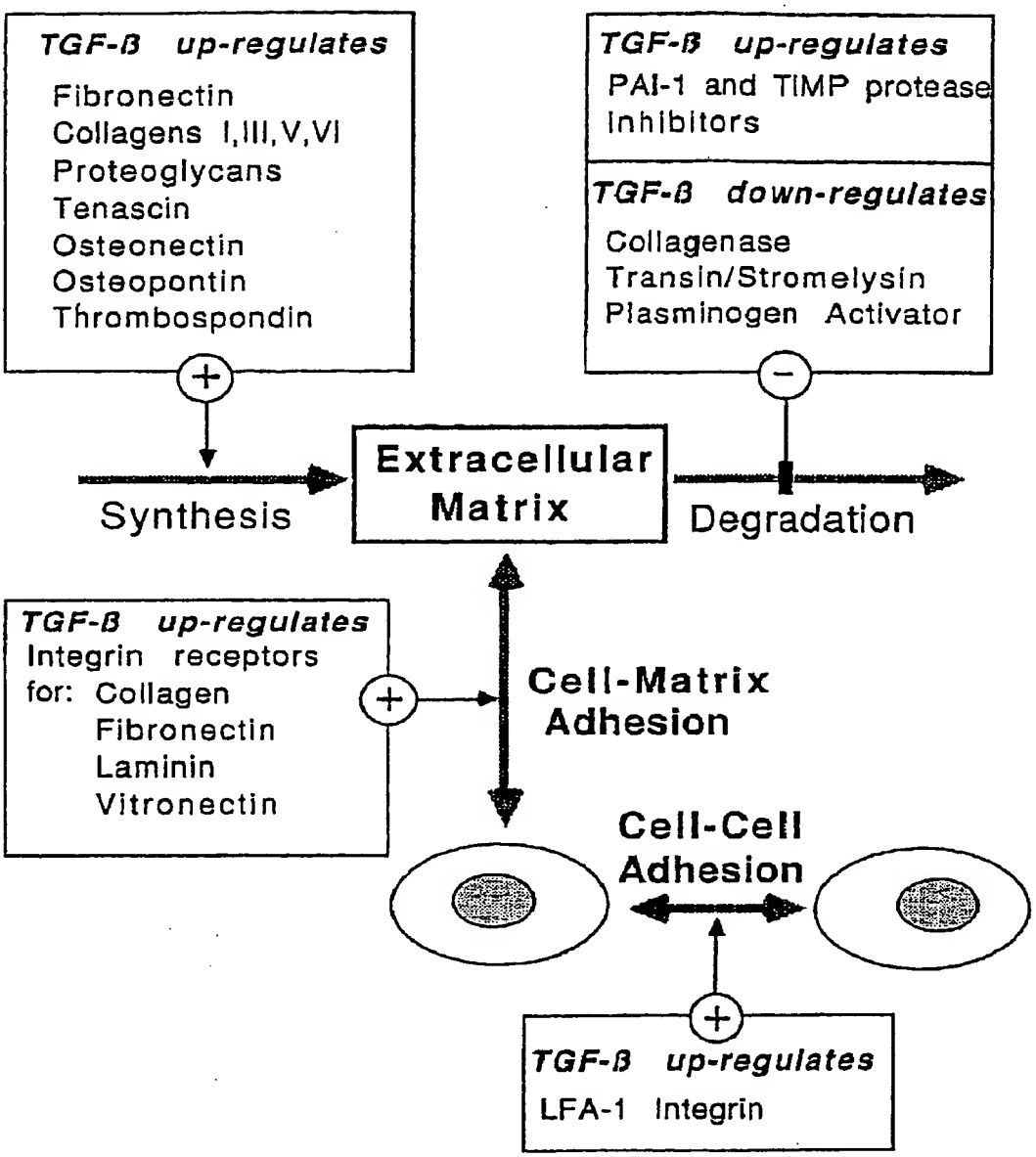

Figure 2 Cell adhesion molecules whose expression is regulated by TGF-β.

chondrogenic differentiation in response to TGF-β1 and 2 (Seyedin et al 1985). Other matrix glycoproteins, whose synthesis is elevated in response to TGF-β1, include osteopontin (Noda et al 1988), osteonectin (Noda & Rodan 1987), tenascin (Pearson et al 1988), thrombospondin (Penttinen et al 1988), and the chondroitin/dermatan sulfate proteoglycans, biglycan (PG I) and decorin (PG II) (Bassols & Massagué 1988).

Elevated levels of mRNA for these proteins are observed within 3–5 hr

of TGF-β addition and are the consequence of elevated transcription of the corresponding genes, as well as increased mRNA stability in response to TGF-β (Ignotz et al 1987; Raghow et al 1987; Rossi et al 1988; Penttinen et al 1988; Dean et al 1988). The relative contribution of these two mechanisms may vary with the cell type. Not only do TGF-β1 and 2 elevate the expression of proteoglycan core proteins, but they also increase the size or total mass of glycosaminoglycan (GAG) chains attached to them (Bassols & Massagué 1988). The composition of GAG chains in the mouse epithelial cell membrane proteoglycan syndecan (Rapraeger 1989), and the overall synthesis of glycosaminoglycans in arterial smooth muscle cells, skin fibroblasts, and growth plate chondrocytes are also up-regulated by TGF-β (Chen et al 1987; Falanga et al 1987). The mechanism by which TGF-β affects the elongation and termination of GAG chains is presently unknown.

The expected net effect of TGF-β action is an accumulation of extracellular matrix. Indeed, this is a major response observed following local injection of TGF-β1 in animals (Roberts et al 1986). In addition, the action of TGF-β may also lead to important qualitative changes in the composition and structure of extracellular matrices. For example, an alteration in the relative proportion of fibronectin and tenascin may lead to changes in cell behavior because of the antagonistic role of these two proteins as mediators of cell adhesion (Chiquet-Ehrismann et al 1988).

CONTROL OF PERICELLULAR PROTEOLYSIS Elevated synthesis of extracellular matrix components is not solely responsible for the net accumulation of extracellular matrix induced by TGF-β. Plasminogen activator inhibitor-1 (PAI-1) and tissue inhibitor of metalloprotease (TIMP), two inhibitors of extracellular matrix degrading enzymes, are strongly up-regulated by TGF-β (Laiho et al 1986, 1987; Lund et al 1987; Edwards et al 1987). The up-regulation of PAI-1 mRNA is due, at least in part, to increased transcription, occurs with faster kinetics (<30 min) than the response of other extracellular matrix components, and can reach up to 50-fold over the basal level. In addition, TGF-β can also decrease the expression of collagenase (Edwards et al 1987), transin/stromelysin (Matrisian et al 1986; Kerr et al 1988), plasminogen activator (Laiho et al 1986), and a thiol proteinase (Chiang & Nilsen-Hamilton 1986).

CONTROL OF CELL ADHESION RECEPTORS The integrins are one of the best characterized families of cell adhesion receptors (Hynes 1987; Ruoslahti & Pierchsbacher 1987). Integrins were examined in detail as targets for TGF-β action after it was observed that TGF-β-treated cells have a higher ability to bind fibronectin and collagen (Ignotz & Massagué 1986). Mouse

thymocytes treated with TGF-β bind more readily to fibronectin-coated tissue culture dishes, and their attachment can be prevented by short synthetic RGD-containing peptides, which indicates that adherence is mediated by integrins (Ignotz & Massagué 1987). Integrins are heterodimeric membrane glycoproteins that consist of one 130–200-kd α subunit and one 90–130-kd β subunit. At least four distinct β integrin subunits exist in human cells, each one able to pair with various α subunits that confer ligand-binding preference to the resulting αβ integrin complex. Many integrins function as adhesion receptors for extracellular matrix components including fibronectin, collagen, laminin, vitronectin, fibrinogen, von Willebrand factor, and other as yet unidentified matrix components. Integrins that have a β2 subunit function as cell-cell adhesion receptors in lymphocytes. Most cell types express various integrins simultaneously, each cell type having a characteristic complement of these receptors.

TGF-β induces marked alterations in the repertoire of integrins expressed in many cell types, primarily by increasing the mRNA levels of individual subunits (Ignotz & Massagué 1987; C. J. Roberts et al 1988; Heino et al 1989; Ignotz et al 1989). TGF-β can alter the expression of all integrin subunits examined to date. These include (*a*) α_1 through α_6 subunits and the β_1 subunit, which combine to generate receptors for fibronectin, collagen, laminin, and other extracellular matrix molecules; (*b*) α_v and β_3 subunits that form a vitronectin receptor; and (*c*) the α_L subunit that combines with β_2 to constitute LFA-1, a cell-cell adhesion receptor that binds to intercellular adhesion molecule ICAM-1 on the surface of other lymphoid cells. Correct assembly of integrins is required for their expression on the cell surface. If some subunits are available in limited amounts, the unassembled excess of other subunits is degraded even before they transit through the Golgi apparatus. Since several integrins may share a common pool of β subunits, the relative change in cell surface integrin levels induced by TGF-β depends not only on the changes in the rate of synthesis of individual subunits, but also on the balance of the various α and β subunits produced by the cell (Heino et al 1989).

The susceptibility of individual integrins to up-regulation by TGF-β depends on the cell type (Heino et al 1989). The expression of a given integrin subunit may not be elevated in a given cell type, or may even be strongly down-regulated, as is the case of the α_3 subunit in MG-63 osteosarcoma cells (Heino & Massagué 1989). Through this set of up- and down-regulatory events TGF-β can alter the repertoire of cell adhesion receptors and the ability of cells to interact with other cells and extracellular matrices.

Control of Cell Phenotype

Initial reports of TGF-β1 influencing cellular differentiation processes (Ignotz & Massagué 1985; Seyedin et al 1985; Masui et al 1986) and the expression of differentiated functions (Rook et al 1986; Hotta & Baird 1986) led to studies showing that the differentiative potential of many cell lineages can be affected by TGF-β in vitro. Table 2 includes a summary of cell lines or primary cell cultures whose differentiation is regulated, positively or negatively, by TGF-β. In addition, the table lists various specialized functions of terminally differentiated cells that are affected by these factors.

General features of the inhibitory action of TGF-β on cell differentiation have been defined in studies on preadipocytes and myoblasts. Mouse 3T3-L1 preadipocytes (Green & Meuth 1974) and rat L_6E_9 skeletal muscle myoblasts (Nadal-Ginard 1978) can be induced to differentiate, respectively, into mature adipocytes and multinucleated myotubes. Differentiation becomes complete within 3–4 days after induction. When these cells are induced to differentiate in the presence of picomolar concentrations of TGF-β, differentiation is blocked (Ignotz & Massagué 1985; Massagué et al 1986; Florini et al 1986). TGF-β1 can be added as late as 30 hr post-induction and still block preadipocyte differentiation. Once 3T3-L1 cells become committed to differentiate, however, they are refractory to the inhibitory action of TGF-β1. Like preadipocytes, L_6E_9 myoblasts go through a critical temporal point after which differentiation will proceed even in the presence of TGF-β. This refractoriness to TGF-β is not due to a loss of receptors since 3T3-L1 adipocytes and L_6E_9 myotubes continue to express TGF-β receptors I and II at levels similar to those of the undifferentiated counterparts, and they continue to show various biochemical responses to TGF-β, such as elevation of extracellular matrix protein expression (Ignotz & Massagué 1985; Massagué et al 1986). Inhibition of differentiation is not secondary to effects of TGF-β1 on cell proliferation, and it is reversible once TGF-β1 is removed (Ignotz & Massagué 1985; Massagué et al 1986; Florini et al 1986). The inhibitory effect of TGF-β on 3T3-L1 and L_6E_9 cell differentiation correlates with a marked alteration in the expression of extracellular matrix proteins and cell adhesion receptors (Ignotz & Massagué 1986, 1987; Ignotz et al 1987). The possible participation of this response in mediating the anti-differentiative action of TGF-β is discussed below.

TGF-β is thought to favor chondrogenesis and osteogenesis, and it exerts positive effects on these cell lineages in vitro (Seyedin et al 1985; Pfeilschifter et al 1987); however, TGF-β can inhibit the expression of osteogenic differentiation markers in some cell lines (Noda & Rodan 1987;

Table 2 Effects of TGF-β1 on cell phenotype

Cell type[a]	Function	Effect of TGF-β[b]	References
Preadipocytes (3T3-L1, Balb/c 3T3, TA1)	Differentiation	−	Ignotz & Massagué 1985; Sparks & Scott 1986; Torti et al 1989
Skeletal muscle myoblasts (L_6, L_8, C2, BC_3H1)	Differentiation	−	Massagué et al 1986; Olson et al 1986; Florini et al 1986
Muscle satellite cells	Differentiation	−	Allen & Boxhorn 1987
Prechondroblasts	Differentiation	+	Seyedin et al 1985
Osteoblastic osteosarcoma (ROS 17/2.8)	Differentiation	+	Pfeilschifter et al 1987
Osteoblasts (MC3T3L1)	Differentiation	−	Noda & Rodan 1987; Rosen et al 1988
Intestinal epithelial cells (IEC-6)	Differentiation	+	Kurokowa et al 1987; Barnard et al 1989
Megakaryocytes	Differentiation	−	Ishibashi et al 1987
Hematopoietic progenitor cells (B6Sut-A, 32D-cl3)	Differentiation associated with proliferation	−	Ohta et al 1987; Keller et al 1988; Ottmann & Pelus 1988
Bronchial epithelial cells	Differentiation markers	+	Masui et al 1986; Jetten et al 1986
Natural killer cells	Cytolysis	−	Rook et al 1986
B-lymphocytes	Ig production	−	Kehrl et al 1986a
Lymphocyte-activated killer cells	Cytokine production	−	Espevik et al 1988
Monocytes	Cytokine production	+	Wahl et al 1987
Macrophages	Respiratory burst	−	Tsunawaki et al 1988
Endothelial cells	Invasion	−	Müller et al 1987
Keratinocytes	Keratinogenesis	+	Reiss & Sartorelli 1987; Mansbridge & Hanawalt 1988
Adrenocortical cells	Steroidogenesis	−	Hotta & Baird 1986; Feige et al 1987
Leydig cells	Steroidogenesis	−	Linn et al 1987; Avallet et al 1987
Granulosa cells	Steroidogenesis	+	Ying et al 1986a
Pituitary cells	FSH production	+	Ying et al 1986b
Adipocytes (TAI-1)	Lipogenesis	−	Torti et al 1989

[a] Continuous cell lines are indicated in parentheses. [b] −, inhibition; +, stimulation.

Rosen et al 1988). These distinct responses might reflect genuine differences between osteogenic cell types. Alternatively, they might reflect a distortion of the normal TGF-β response by the constraints of in vitro culture conditions. Of course, the latter consideration also applies to the other

differentiative processes that are affected by TGF-β in vitro. In vitro systems may demonstrate the existence of biochemical mechanisms that couple TGF-β to the control of cell phenotype, but in vivo studies are necessary to establish the response that activation of these mechanisms by TGF-β will ultimately induce in the physiologic setting.

Physiology and Pathology

EMBRYOGENESIS TGFs-β are expressed throughout embryogenic development, and their receptors are ubiquitously distributed. This, and the ability of TGF-β to control DNA replication, cell differentiation, cell adhesion, and extracellular matrix layout suggest a broad role for TGF-β in the generation and modification of extracellular cues that guide the morphogenic events of embryogenesis. Thus far, however, the evidence that TGFs-β play a critical role in embryogenesis remains correlative in nature.

Expression of TGFs-β is high in sites undergoing intense development and morphogenesis. These include sites of chondrogenesis and osteogenesis: long bone growth plates or somites developing into vertebrae (Ellingsworth et al 1986; Heine et al 1987; Sandberg et al 1988), hematopoietic organs (Ellingsworth et al 1986; Wilcox & Derynck 1988), and epithelial/mesenchymal interfaces (Lehnert & Akhurst 1988; Pelton et al 1989). That early embryo tissues are responsive to TGF-β has been demonstrated by addition of mammalian TGF-β1 (together with fibroblast growth factor) or TGF-β2 to ectoderm explants from *Xenopus laevis* embryos. These additions promote the generation of mesoderm (Kimelman & Kirschner 1987; Rosa et al 1988).

TISSUE REPAIR, INFLAMMATION, AND ANGIOGENESIS TGFs-β stored at high levels in platelets (Assoian et al 1983; Cheifetz et al 1987) or expressed in activated monocytes and macrophages (Assoian et al 1987; Tsunawaki et al 1988) can be physiologically delivered to sites of wound healing or inflammation. The constant remodeling and recycling of bone matrix and marrow, or their repair, are accessible to control by TGFs-β that are abundantly present in these tissues (Seyedin et al 1985; Thompson et al 1989). The activity of TGF-β as a promoter of extracellular matrix deposition and a regulator of cell migration and development probably plays a major influence in these processes. In addition, TGF-β1 is an extremely potent chemoattractant for monocytes (Wahl et al 1987) and, to a lesser extent, fibroblasts (Postlethwaite et al 1987). TGF-β1 may attract these cells to sites of inflammation and repair. Indeed, administration of TGF-β1 into wound chambers, subcutaneously, or to incisional wounds, stimulates the accumulation of granulation tissue and cellularization of the

wound bed and accelerates the wound healing response in general (Sporn et al 1983; Roberts et al 1986; Mustoe et al 1987).

Directly or through other cells that it attracts and stimulates, TGF-β1 can induce formation of new blood vessels in vivo (Roberts et al 1986). This response might seem paradoxical given the strong growth inhibitory effect of TGF-β1 and TGF-β3 on endothelial cell monolayer cultures (Frater-Schröder et al 1986; Heimark et al 1986; Cheifetz et al 1990). It has been noted, however, that under certain culture conditions endothelial cells are not growth-inhibited by TGF-β1 and tend to organize into tubular structures reminiscent of an angiogenic process (Majack 1987; Madri et al 1988).

IMMUNOSUPPRESSION The observation that glioblastoma is frequently accompanied by immunosuppression led to the isolation of TGF-β2 as a glioblastoma cell-derived suppressor of T cells in vitro (Wrann et al 1987; de Martin et al 1987). Both TGF-β1 and TGF-β2 exhibit activities in vitro that are consistent with an immunosuppressive action in vivo. These include the antiproliferative effects on thymocytes, T- and B-lymphocytes described above, as well as multiple negative effects on differentiated functions of B-lymphocytes, natural killer cells, lymphocytes activated killer cells, and macrophages, as described in Table 2 and references therein. The importance of the TGF-β contribution to the pathophysiology of immunosuppression remains to be determined.

FIBROSIS A localized excess of TGF-β activity in tissues could lead to an unbalanced deposition of extracellular matrix and contribute to a variety of fibrotic disorders. A case in point is the condition known as proliferative vitreoretinopathy (PVR) (Connor et al 1989). PVR occurs in 10% of eyes that undergo surgery for retinal detachment, and it leads to excessive intraocular fibrosis and blindness. The levels of TGF-β activity in PVR are threefold higher than normal; this activity appears to correspond to TGF-β2. Injection of TGF-β into the vitreous cavity of test animals produces a PVR condition (Connor et al 1989). Detailed studies of other fibrotic disorders might reveal a wider role of TGF-β in these disorders.

ONCOGENESIS Unrestricted cell growth caused by the lack of TGF-β growth inhibitory activity is perhaps the most important of the possible consequences that would derive from a pathological loss of TGF-β function. Such loss could be caused by defects in TGF-β expression or activation, or defects at the TGF-β receptor or post-receptor levels. The level of TGF-β expression varies widely among normal cell lines, however, and many normal cell lines are unable to activate the latent TGF-β that they produce in vitro. These facts generate some uncertainty in assessing

to what extent a low level of expression or activation of TGF-β in a given tumor-derived cell type might contribute to the oncogenic transformation of these cells. Similar limitations exist in determining the significance of a lack of growth inhibitory response to TGF-β in tumor cells since not all normal cells are growth inhibited by these factors. It is worth noting, however, that rat liver epithelial cells (McMahon et al 1986) and human retinoblastoma cells (Kimchi et al 1988) do not respond to TGF-β, whereas their normal counterparts are growth inhibited by this factor.

The loss of TGF-β receptors is a highly unusual event among some 150 cell lines and primary cells examined, normal or transformed (Wakefield et al 1987; Massagué et al 1990). Human retinoblastoma cells are a notable exception to the ubiquitous distribution of TGF-β receptors (Kimchi et al 1988). Eight independently derived retinoblastoma cell lines are not growth inhibited by TGF-β and lack detectable TGF-β receptors I or II (see below), in contrast to normal fetal retina cells, which display the full repertoire of TGF-β receptors and are growth inhibited by this factor. It has been suggested that the lack of functional TGF-β receptors in retinoblastoma cells, whose primary defect lies in the RB gene, may permit these tumor cells to escape growth suppression by TGF-β in the retina (Kimchi et al 1988). It remains to be determined how widespread the absence of TGF-β receptors is among other tumor cell types that have a defective RB gene locus, and which, if any, mechanism might link RB function to expression of TGF-β receptors. Other cell lines that have no TGF-β receptors I or II detectable by affinity-labeling techniques include PC12 rat pheochromocytoma cells and N2A mouse neuroblastoma cells (Kimchi et al 1988).

TGF-β RECEPTORS AND OTHER BINDING PROTEINS

Hormonally active polypeptides are believed to act on target cells by binding avidly and specifically to integral membrane proteins that are coupled to cytoplasmic signal transducers. TGF-β binds with high affinity to the surface of many cell types (Wakefield et al 1987). Covalent tagging of cells with radiolabeled TGFs-β using cross-linking agents or a photoreactive TGF-β1 analogue reveals that binding is mediated by several coexisting membrane proteins (Massagué & Like 1985; Cheifetz et al 1986). Some of these proteins are likely to mediate TGF-β action, whereas others may fulfill a different function.

Two glycoproteins (receptors I and II) of 53 and 70–100 kd, respectively, and a membrane proteoglycan, designated betaglycan (formerly type III

receptor) have been identified as the most widespread high-affinity TGF-β-binding components. Receptors I and II bind TGF-β1 with higher affinity than betaglycan, and are ubiquitously present at low levels in mammalian and avian cells, with the exceptions mentioned above. Betaglycan is broadly distributed, but it is not detectable in various cell types that respond to TGF-β including skeletal muscle myoblasts, hematopoietic progenitor cells, and vascular endothelial cells (Massagué et al 1986, 1990; Ohta et al 1987; Segarini et al 1989; Goodman & Majack 1989). Properties of these TGF-β-binding components are listed in Table 3. A detailed review of their cellular distribution and other properties has been recently presented (Massagué et al 1990).

Isolation of TGF-β receptor cDNAs has been an elusive goal. Therefore, formal identification of the TGF-β receptor based on expressing its cDNA in receptor-defective cells has not yet been achieved. The cellular distribution of TGF-β receptors and, in particular, the isolation of TGF-β-resistant cell mutants, however, have provided substantial evidence implicating receptor types I and II as components of the signal-transducing TGF-β receptor complex.

TGF-β Receptors

RECEPTOR COMPONENTS I AND II The possibility that receptors I or II are involved in mediating TGF-β action was first suggested by the fact that TGF-β1 controls cell differentiation, collagen, and fibronectin expression in skeletal muscle myoblasts that express receptors I and II, but no beta-

Table 3 Cell surface TGF-β-binding proteins

	Type I	Type II	Betaglycan	GH_3 protein
Probable function	Signal transduction	Signal transduction	TGF-β storage or transport	Unknown
Ligands	TGFs-β	TGFs-β	TGFs-β	TGFs-β, activins inhibins
Glycoprotein, kd[a]	53 (65)[b]	70–100 (85–110)	200–400	60 (70–74)
Deglycosylated, kd[c]	48	55–75	100–120	60
Carbohydrate type[c]	N-linked	N-linked	Glycoaminoglycans and N-linked glycans	Unknown
K_D for TGF-β1	5–50 pM	5–50 pM	30–300 pM	90 pM
Order of affinities	β1 ~ β3 > β2	β1 > β3 > β2	β1 ~ β2 ~ β3	β1 > β2 ≥ Act ~ Inh
Binding sites/cell	Up to 4,000	Up to 4,000	Up to 10^5	2,700
Distribution	Ubiquitous	Ubiquitous	Broad	GH_3 pituitary cells

[a] Estimated molecular weight of the membrane glycoprotein form. [b] Molecular weight of the affinity-labeled complex is shown in parentheses. [c] Deduced by treatment of the protein with trifluoromethanesulfonic acid, N-glycanase, O-glycosidase, chondroitinase ABC and/or heparitinase.

glycan (Massagué et al 1986), and inhibits proliferation of murine hematopoietic progenitor cells expressing receptor type I as the only detectable TGF-β-binding component (Ohta et al 1987).

In all avian and mammalian cell types examined, the type I TGF-β receptor component is a glycoprotein of 53 kd, a value estimated by subtraction of the mass contributed by reduced TGF-β to the 65 kd affinity-labeled receptor complex (Cheifetz et al 1986). This receptor component contains an N-linked carbohydrate, which is not required for cell surface expression of the receptor, or for TGF-β binding, as shown after inhibition of co-translational glycosylation with tunicamycin (Cheifetz et al 1988a). Characteristically, the type I receptor discriminates between various forms of TGF-β in receptor competition assays. The order of relative affinities is TGF-β1 ~ TGF-β3 > TGF-β1.2 > TGF-β2, with a 10–20 fold difference between the affinity constants for TGF-β1 and TGF-β2 (Cheifetz et al 1987, 1988b, 1990; Segarini et al 1987).

The type II receptor shares many of the characteristics of the type I receptor. It is also a glycoprotein with N-linked carbohydrate that is not required for cell surface expression or for ligand binding (Cheifetz et al 1986, 1987, 1988a). This receptor can discriminate between the various forms of TGF-β with a similar, albeit not identical, range of affinities as the type I receptor. Despite these similarities, comparative peptide mapping experiments indicate that the binding domains of these receptors are distinct. Unlike the type I receptor, the type II receptor shows a great variability in size, from an estimated 70 to 85 kd in most mammalian cells to 100 kd in chick embryo fibroblasts (Cheifetz et al 1986). The size variability in mammalian cells appears to be largely the result of differences in glycosylation. However, the deglycosylated polypeptide from chick embryo fibroblasts is 20 kd larger than the corresponding mammalian product (S. Cheifetz, personal communication).

Receptor I is expressed in most of the over one hundred cell lines and tissues tested by affinity-labeling procedures (Massagué et al 1990). Several murine hematopoietic progenitor cell lines (B6SUt-A, 32D-C13) that are growth-inhibited by TGF-β are among the very few known cases in which no clearly detectable type II receptor is co-expressed with the type I receptor (Ohta et al 1987). These cells respond to TGF-β1, 1.2, and 2 with an order of potencies that parallels the order of affinities of these factors for receptor I (Ohta et al 1987; Cheifetz et al 1988b). Human and bovine vascular endothelial cells, which express receptors I and II, are also growth-inhibited more potently by TGF-β1 than TGF-β2 (Jennings et al 1988), but in this case the difference appears to result from a more rapid inactivation of TGF-β2 (Cheifetz et al 1990). Most other mammalian cell lines tested show similar sensitivity to various forms of TGF-β, whether the

cells co-express receptors I and II alone or with betaglycan. Recent binding saturation studies with Mv1Lu mink lung epithelial cells have suggested the presence of a population of receptors I and II that has high affinity for TGF-β1, 2, and 3 and binds these factors in the concentration range that is sufficient for a maximal growth inhibitory response. The other, more abundant, receptor population has the order of affinities TGF-β1 > TGF-β3 > TGF-β2 observed in receptor competition experiments using higher ligand concentrations (Cheifetz et al 1990). The biological role of these spare receptors is unknown.

EXPERIMENTALLY INDUCED RECEPTOR MUTANTS The phenotype of various experimentally induced TGF-β-resistant cell mutants derived from Mv1Lu cells provides the most persuasive evidence to date implicating TGF-β receptors I and II in signal transduction (Boyd & Massagué 1989; M. Laiho et al submitted). Parental Mv1Lu cells are potently (ED_{50} = 1pM) growth arrested by TGF-β and express receptors I and II, as well as betaglycan. The mutant cell clones have lost all known TGF-β responses including growth inhibition. With high frequency the mutations affect receptors I and II; however, betaglycan is not affected (Boyd & Massagué 1989; Laiho et al 1990). Some mutant clones show normal binding to the type II receptor, but are defective in type I receptor (R mutants), whereas other clones (S mutants) express all receptors in a profile identical to the parental cell line. A third group of mutants is defective in both the type I and type II receptors (DR mutants), which are either undetectable or present in very low levels with the type II receptor and have, in addition, decreased electrophoretic mobility. Analysis of somatic cell hybrids has indicated that all these mutant phenotypes are recessive (Boyd & Massagué 1989; M. Laiho et al submitted). The DR phenotype could be due to independent mutational events affecting both receptors, but a more attractive possibility is that receptors I and II interact with each other so that mutations affecting the expression or structure of one receptor component may impair the expression or function of the other.

A TGF-β RECEPTOR MODEL The phenotype of these TGF-β-resistant cell mutants implicates receptors I and II as components of the receptor complex that mediates multiple TGF-β responses including growth inhibition, extracellular matrix protein up-regulation, and the morphological response. Since receptors I and II are also present in cell lines that are not growth-inhibited by TGF-β, the different responsiveness of cells to these factors is probably determined by the ability of the receptor to couple with different signaling pathways in each cell type. A model in which a single receptor complex with two TGF-β-binding subunits mediates multiple actions of TGF-β prevails at present over the hypothesis that the different

responses to TGF-β are mediated by multiple unrelated receptor types. Given that TGFs-β are dimeric, it is possible that a single TGF-β molecule could simultaneously interact with the two receptor components I and II.

TGF-β RECEPTOR REGULATION Loss of individual TGF-β receptors in retinoblastoma cells (Kimchi et al 1988) suggests the existence of mechanisms that regulate TGF-β receptor expression. The available evidence, however, suggests that the expression and dynamics of TGF-β receptors are in general not highly regulated. TGF-β receptors do not appear to display the acute regulation by homologous and heterologous ligands that is commonly seen in receptors for some other growth factors. This is despite the fact that receptor-bound TGF-β is rapidly internalized and degraded (Frolik et al 1984; Massagué & Kelly 1986). TGF-β binding increases during differentiation of murine embryonal carcinoma cells (Rizzino 1987) and during T cell activation (Kehrl et al 1986b). Adrenocortical cells show increased TGF-β binding to all three receptor forms following stimulation with adrenocorticotrophic hormone (Cochet et al 1988). Loss of betaglycan, or gain of betaglycan with loss of receptors I and II have been described, respectively, in smooth muscle cells and vascular endothelial cells as a function of cell density (Goodman & Majack 1989; Müller et al 1987). Other investigators, however, have not observed effects of cell density on TGF-β receptors in vascular endothelial cells (Segarini et al 1989). Changes in receptor profile as a function of cell density do not appear to be a general phenomenon (S. Cheifetz, personal communication).

Betaglycans: Proteoglycans with High Affinity for TGF-β

In many cell lines, the most abundant cell-surface TGF-β-binding component is betaglycan (Massagué 1985; Massagué & Like 1985; Cheifetz et al 1986; Fanger et al 1986). Betaglycan is heterogeneous in nature and typically runs on SDS-polyacrylamide gels as a broad band with an average mass of 280–330 kd. One of the remarkable characteristics of betaglycan is that it is an integral membrane proteoglycan consisting of approximately 200 kd of glycosaminoglycan (GAG) chain mass and 10 kd of N-linked glycans attached to a heterogeneous core polypeptide of 100–120 kd (Segarini & Seyedin 1988; Cheifetz et al 1988a). Betaglycan may contain only one class of glycosaminoglycan chains, but most betaglycan forms contain heparan sulfate as well as chondroitin sulfate GAG chains in a proportion that varies in different cell types (Cheifetz & Massagué 1989). In some cell lines, betaglycan appears to be part of a disulfide-linked complex (Massagué 1985; Fanger et al 1986). In contrast to receptors I and II, betaglycan shows similar affinity for TGF-β1, 2, and 3 in many cell lines

tested (Cheifetz et al 1987, 1988b, 1990; Cheifetz & Massagué 1989), but one study suggests the presence of a mixed receptor population with different affinities for TGF-β1 and TGF-β2 in some cell lines (Segarini et al 1987).

Experiments with cell mutants defective in GAG synthesis (Cheifetz & Massagué 1989) and the use of GAG-degrading enzymes (Segarini & Seyedin 1988; Cheifetz et al 1988a) have shown that the TGF-β binding site resides in the betaglycan core protein and that the GAGs are not required for TGF-β binding or functional expression of betaglycan on the cell surface. The growth inhibitory response to TGF-β is similar in parental cells and the GAG-defective mutants.

Betaglycan may belong to the recycling receptor class since it appears to be internalized with TGF-β, but this does not lead to an acute down-regulation of cell surface betaglycan level (Massagué & Kelly 1986). Betaglycan forms that lack the membrane anchor are released by cultured cells into the medium and are found in low amounts in serum and in extracellular matrices (Andres et al 1989). The soluble forms of betaglycan also differ from the membrane forms in the electrophoretic mobility of their 100–110 kd core devoid of GAG chains. It is not known whether the soluble form of betaglycan is derived from the membrane bound form by a hydrolytic process, is the product of alternative mRNA splicing, or is encoded by a separate gene.

Betaglycan was the first TGF-β-binding component to be identified as a putative TGF-β receptor based on its relative abundance in 3T3 fibroblasts and the correlation between biological potency and betaglycan affinity for various TGF-β forms (Massagué & Like 1985; Cheifetz et al 1987). The TGF-β signal transducing activity is now ascribed to receptors I and II. Betaglycan might, however, be involved in ligand presentation to these receptors. Given its structural features, relative abundance, and secretory nature, betaglycan could function as a reservoir or clearance system for bioactive TGF-β. By analogy with membrane proteoglycans that may participate in cell adhesion and recognition (Rapraeger 1989), it is possible that betaglycan could also have such adhesive functions.

Activin-Binding Proteins

Betaglycan and the TGF-β receptors I and II do not recognize activins or inhibins. A TGF-β binding protein that cross-reacts with inhibins and activins, however, has been identified by affinity-labeling of GH_3 rat pituitary tumor cells (Cheifetz et al 1988c). This protein has an estimated molecular weight of 60 kd (after subtracting the mass of cross-linked TGF-β monomer). The affinity of this receptor for TGF-β1 ($K_d = 90$ pM) is higher than for TGF-β2, activin, or inhibin. These values parallel the

relative potency of these factors to regulate FSH production in rat pituitary cell cultures (Ling et al 1986; Ying et al 1986a). The presence of this receptor in primary pituitary cells could not be determined, and its biological significance remains to be determined.

Activin receptors (K_d = 30 pM, 3,200/cell) are present in murine F5–5 erythroleukemia cells (Hino et al 1989). Binding of activin-A to these receptors is not inhibited by TGF-β1, which is in agreement with the inability of TGF-β1 to mimic activin action on erythroblastic cells (Eto et al 1987). Cross-linking of these receptors with radiolabeled activin yields two major labeled products of 67 and 76 kd, respectively; a pattern that is reminiscent of that for the affinity-labeled TGF-β receptors I and II. This resemblance raises the possibility that receptors for the activins and inhibins, the TGFs-β and, perhaps, the other factors of this large family have structural features in common.

An activin-binding protein isolated from rat ovary by activin-affinity chromatography is follistatin (Nakamura et al 1990). Follistatin is a soluble 35-kd glycoprotein that was initially identified by its ability to inhibit FSH production by the pituitary gland. Follistatin binds activin with high affinity (K_d = 590 pM) and blocks its FSH-inducing activity in the pituitary (Nakamura et al 1990). Thus the action of activin appears to be tightly regulated by two types of antagonists, the inhibins and follistatin.

MECHANISM OF TGF-β ACTION

Based on the structural and functional homologies of the members of the TGF-β superfamily, it is likely that these factors interact with a family of structurally related receptors. The unique range of biological activities displayed by these factors suggests the possibility that their signal transduction mechanisms might be quite distinct from others presently known.

The Cytoplasmic Response

The primary signal transduction mechanism of TGF-β receptors is not known. Several efforts to determine whether enzymatic activities and second messengers that are directly involved in signaling by other hormone and growth factor receptors might be coupled to TGF-β receptors have met with negative results (Like & Massagué 1986; Chambard & Pouyssegur 1988; Fanger et al 1986; J. Massagué, unpublished work). Interestingly, agents that increase cAMP accumulation block induction of c-*sis* by TGF-β1 in vascular endothelial cells (Daniel et al 1987). Inhibition of TGF-β1-induced c-*sis* expression by cholera and pertussis toxins has been reported in AKR-2B fibroblasts (Murthy et al 1988; Howe & Leof 1988; Howe et al 1990). These toxins catalyze ADP-ribosylation and inactivation of

guanine nucleotide-binding proteins (G proteins), which are involved in coupling certain hormone receptors to effector molecules. Both toxins can inhibit the mitogenic response of AKR-2B fibroblasts to TGF-β1, but do not alter other TGF-β responses or TGF-β1 binding to receptors (Howe et al 1990).

Activation of glycolysis, amino acid uptake, intracellular calcium levels, and phosphatidyl inositol turnover have been observed in rat fibroblasts in response to TGF-β (Boerner et al 1985; Inman & Colowick 1985; Muldoon et al 1988). The effect of TGF-β1 on these parameters does not show the rapid kinetics characteristic of the response to certain hormones and pharmacologic agents. Furthermore, the elevation of cytosolic calcium and accumulation of inositol triphosphate appear to require gene transcription (Muldoon et al 1988), which indicates that these cytoplasmic responses are not directly coupled to TGF-β receptors. TGF-β1 can also stimulate prostaglandin E_2 production in lung fibroblasts (Diaz et al 1989) and cultured mouse calvaria (Tashjian et al 1985).

The Nuclear Response

A basis for the diversity, sometimes disparity, of the biological responses induced by TGF-β in different cell types, or in the same cell type under different conditions, can be gleaned from studies on the control of gene expression by these factors. The expression of an array of genes related to growth control, differentiation, and cell adhesion can be markedly altered within 0.5 to 3 hr of addition of TGF-β to cells. Although post-transcriptional events (Raghow et al 1987; Penttinen et al 1988; Morrone et al 1989) may contribute to this response, TGF-β frequently controls gene expression at the transcriptional level, either positively (Ignotz et al 1987; Dean et al 1988; Lund et al 1987; Rossi et al 1988; Machida et al 1988; Kerr et al 1988; Thompson et al 1988; Kim et al 1989a, 1990) or negatively (Kerr et al 1988, 1990; Pietenpol et al 1990). The nature of the nuclear response to TGF-β is quite complex, but the accumulating evidence leads to the following conclusions.

First, TGF-β can regulate different transcriptional control elements. Furthermore, different TGF-β-responsive elements can coexist in the same gene promoter. For example, transcriptional activation by TGF-β1 is mediated by a NF-1 binding site in the collagen α2(I) gene promoter (Rossi et al 1988), by multiple AP-1 binding sites in the TGF-β1 gene promoter (Kim et al 1990), and by three distinct elements in the fibronectin gene promoter. One of the elements in the fibronectin gene promoter is a NF-1 binding site; its deletion results in only a partial decrease in transcriptional activation by TGF-β (D. C. Dean, S. Bourgeois, personal communication).

Second, cell-specific determinants dictate the type of response of a par-

ticular gene to TGF-β. For example, transcription of c-*jun* and *junB* can be elevated by TGF-β, but whether one or both of these genes will respond, and with what kinetics, depends on the cell type (Pertovaara et al 1989; Kim et al 1990; Li et al 1990). A survey of numerous integrins has shown that expression of most of them can be elevated by TGF-β1 action, but only a subset of the integrins expressed in a given cell type may respond to this factor (Heino et al 1989; Ignotz et al 1989). Expression of the α_3 integrin subunit in MG-63 osteosarcoma cells is inhibited by TGF-β1 even though TGF-β1 elevates expression of this integrin subunit in other cell types and elevates expression of other integrins in MG-63 cells (Heino & Massagué 1989). These responses appear to be at the transcriptional level (M. Bosenberg et al, unpublished), and it will be interesting to determine how TGF-β controls positive or negative regulatory elements in each cell type.

Third, some of the genes whose expression is regulated by TGF-β encode transcription factors (c-*jun*, *junB*, *fos*, *myc*), growth factors (PDGF-A, PDGF-B), or other products that can, in turn, generate a secondary nuclear response. Several of the proliferative and differentiative effects of TGF-β probably follow from this secondary wave of nuclear events.

Growth Suppression Mechanisms

Various mechanisms have been proposed to explain the growth inhibitory action of TGF-β. Several reports have tentatively attributed this action to the ability of TGF-β to decrease expression of mitogen receptors, e.g. EGF receptors in NRK rat fibroblasts (Assoian 1985), or PDGF receptors in bone marrow fibroblasts and 3T3 fibroblasts (Bryckaert et al 1988; Gronwald et al 1989). These effects may contribute to the growth-inhibitory response in some cell types, but evidence argues that they do not represent a general mechanism of growth inhibition by TGF-β. Thus EGF-induced proliferation of lung epithelial cells and lung fibroblasts is arrested by TGF-β1 without altering EGF binding or an array of EGF-induced growth-related responses including activation of plasma membrane H^+/Na^+ antiport, induction of c-*fos* and c-*myc* expression, activation of protein kinase C, or phosphorylation of ribosomal protein S6 (Like & Massagué 1986; Chambard & Pouyssegur 1988 and personal communication). One report proposed that inhibition of NRK fibroblast proliferation is secondary to the action of collagen overproduced in response to TGF-β1 (Nugent & Newman 1989). Accumulation of a collagen-rich extracellular matrix in response to TGF-β1 may affect the long term proliferative capacity of these cells, but the rate of collagen accumulation in fibroblasts is too slow to mediate rapid effects on the cell cycle.

Other approaches have focused on identifying cell cycle events that are directly controlled by TGF-β action. TGF-β1 has been shown to inhibit the expression of certain growth-related genes, particularly c-*myc*, in endothelial cells, colon carcinoma cells, keratinocytes, and breast carcinoma cells (Takehara et al 1987; Fernandez-Pol et al 1987; Coffey et al 1988; Mulder et al 1988). TGF-β1 inhibits c-*myc* gene transcription in mouse keratinocytes stimulated by EGF (Coffey et al 1988; Pietenpol et al 1990). This effect is involved in the growth inhibitory response of keratinocytes to TGF-β (Pietenpol et al 1990). No negative response of c-*myc* has been observed in fibroblasts whose proliferation is blocked by TGF-β1 (Chambard & Pouyssegur 1988; Sorrentino & Bandyopadhyay 1989). Although TGF-β1 can increase the expression of c-*fos*, c-*jun* and *junB*, these responses do not appear to correlate with effects on cell proliferation (Pertovaara et al 1989; Kim et al 1990; Heino & Massagué 1990).

TGF-β inhibits cell cycle progression by lengthening or arresting the G1 phase (Shipley et al 1985; Nakamura et al 1985; Heimark et al 1986; P. Lin et al 1987). In Mv1Lu lung epithelial cells, TGF-β1 acts by interfering with late G1 events (Laiho et al 1990). Significant among these events is phosphorylation of the retinoblastoma gene product, RB. The retinoblastoma gene encodes a product with presumptive growth suppressor activity (Dryja et al 1985; Friend et al 1986; Fung et al 1987; Lee et al 1987). In normal cells, RB is expressed throughout the cell cycle, but exists in multiple phosphorylated forms that are specific for certain phases of the cycle (Ludlow et al 1989; DeCaprio et al 1989; Buchkovich et al 1989; Chen et al 1989). It is thought that the cell cycle regulatory (suppressive) activity of RB is regulated by cell cycle-dependent phosphorylation and dephosphorylation events (Buchkovich et al 1989; DeCaprio et al 1989; Ludlow et al 1990). Underphosphorylated RB is the form with presumed growth suppressive activity since it is prevalent in G1 and in growth-arrested cells (DeCaprio et al 1989; Buchkovich et al 1989; Chen et al 1989) and is selectively bound by the transforming protein of SV40 virus T antigens which might inactivate it (Ludlow et al 1989).

Studies based on these new insights have shown that TGF-β1 added to lung epithelial cells in mid to late G1 rapidly prevents the phosphorylation of RB scheduled for this time point and arrests cells in cycle (Laiho et al 1990). Furthermore, expression of T antigen in Mv1Lu cells does not prevent the effect of TGF-β on RB phosphorylation, but prevents the growth inhibitory response presumably by blocking the growth suppressive activity of unphosphorylated RB. The extent of inhibition of RB phosphorylation induced by TGF-β1 in several cell lines (A549 lung adenocarcinoma, MG-63 osteosarcoma, BSC-1 monkey kidney epithelial cells) is proportional to the intensity of their growth inhibitory response to TGF-

β1. These findings suggest that TGF-β1 and RB function in a common growth inhibitory pathway in which TGF-β1 acts to retain RB in the underphosphorylated, growth suppressive state.

Differentiation Control Mechanisms

The events that commit cells to terminal differentiation have begun to be defined. A family of myogenic differentiation genes, including *MyoD1*, *myogenin*, *Myf-5* and *myd* (Davis et al 1987; Pinney et al 1988; Wright et al 1989; Braun et al 1989), are expressed at high levels during the differentiation process, and induce cell commitment to a muscle phenotype. The MyoD1, myogenin and Myf-5 products have a domain with predicted homology to the helix-loop-helix motif of Myc. This structure is implicated in protein dimerization (Davis et al 1987; Wright et al 1989; Braun et al 1989), in binding to certain muscle gene enhancers (Lassar et al 1989a), and in transcriptional activation of muscle-specific genes (Weintraub et al 1989). Expression of myogenin differentiation genes might be the subject of regulation by factors that control myogenic differentiation, such as TGF-β1. Indeed, TGF-β1 has been shown to repress transcription of *MyoD1* in 23A2 mouse myoblasts (Vaidja et al 1989) and to prevent elevation of myogenin mRNA in L_6E_9 rat myoblasts (Heino & Massagué 1990). These responses may relate to the ability of TGF-β1 to up-regulate Jun expression since overexpression of v-Jun (P. Vogt & H. Su personal communication) or its associated protein, Fos (Lassar et al 1989b), are incompatible with myogenic gene expression.

Given the prominent role that *myogenin* plays in myoblast differentiation (Wright et al 1989), the ability of TGF-β1 to prevent up-regulation of myogenin mRNA is likely to participate in the inhibition of L_6E_9 myoblast differentiation by this factor; however, additional mechanisms may be involved in the anti-myogenic action of TGF-β1. This possibility is suggested by the observation that fibroblasts rendered competent to differentiate by forced expression of *MyoD1* or *myogenin* fail to differentiate in the presence of TGF-β1 (Vaidja et al 1989; Heino & Massagué 1990). The nature of the alternative mechanism has been suggested by the observation that L_6E_9 myoblasts respond to TGF-β1 with a marked elevation of type I collagen synthesis and deposition in the matrix (Massagué et al 1986; Ignotz et al 1987), and L_6E_9 myoblasts plated on a collagen-rich matrix do not differentiate even though their myogenin mRNA level increases several-fold (Heino & Massagué 1990). Cell adhesion is a determinant of L6 myoblast differentiation, as shown by the ability of an anti-integrin antibody to block this differentiation process (Menko & Boettiger 1987). Thus inhibition of myoblast differentiation by TGF-β1 appears to be accomplished by two mechanisms acting in concert. One of these mech-

anisms leads to a block in the expression of a myogenic differentiation gene, such as *myogenin* in L_6E_9 cells. The other mechanism is likely to involve TGF-β-induced changes in cell adhesion that either block the action of myogenic differentiation gene products or prevent the function of other as yet unknown components of the myogenic differentiation pathway.

PROSPECTS

The TGF-β gene superfamily undoubtedly will be more complex than we presently know it. Systematic searches for TGF-β-related factors as well as serendipitous findings will soon outdate the information in Table 1. Current work is also aimed at gaining an understanding of the mechanisms that regulate expression of the various TGF-β-related factors, control activation of their latent forms, and their storage and clearance after release. This information is required to exploit the complex biology of these factors for therapeutic purposes. The spectrum of unique actions of the TGFs-β suggests a plethora of potential useful applications in medicine, but more knowledge and a better understanding of the biological and chemical properties of these factors is needed to use them effectively.

Some of the major tasks that lie ahead relate to the elucidation of the mechanisms of action of TGF-β-related factors and the determinants of cellular responsiveness to them. How can TGF-β regulate diverse transcriptional control factors and orchestrate a distinct pleiotropic nuclear response in each cell type? If, as the emerging evidence indicates, the components that drive the cell cycle are so remarkably conserved in organisms from yeast to humans, how can cells vary so much in their proliferative response to TGF-β? Are the differences dictated by unique sets of biochemical components that couple TGF-β receptors to gene expression and cell cycle control mechanisms in each cell type? The identification of the primary structures of the TGF-β receptors is likely to be accomplished soon. This should help identify the signal transduction components, and components beyond these, in the pathway of TGF-β action. We anticipate a great deal of excitement as the study of the TGFs-β, one of the most fascinating networks of intercellular communication by polypeptide factors, unfolds.

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Literature Cited

Akhurst, R. J., Fee, F., Balmain, A. 1988. Localized production of TGF-β mRNA in tumor promoter-stimulated mouse epidermis. *Nature* 331: 363–65

Allen, R. E., Boxhorn, L. K. 1987. Inhibition of skeletal muscle satellite cell differentiation by transforming growth factor-beta. *J. Cell Physiol.* 133: 567–72

Andres, J. L., Stanley, K., Cheifetz, S., Massagué, J. 1989. Membrane-anchored and soluble forms of betaglycan, a polymorphic proteoglycan that binds transforming growth factor-β. *J. Cell Biol.* 109: 3137–45

Antonelli-Orlidge, A., Saunders, K. B., Smith, S. R., D'Amore, P. A. 1989. An activated form of transforming growth factor-β is produced by cocultures of endothelial cells and pericytes. *Proc. Natl. Acad. Sci. USA* 86: 4544–48

Assoian, R. K. 1985. Biphasic effects of type β transforming growth factor on epidermal growth factor receptors in NRK fibroblasts. *J. Biol. Chem.* 260: 9613–17

Assoian, R. K., Komoriya, A., Meyers, C. A., Miller, D. M., Sporn, M. B. 1983. Transforming growth factor-beta in human platelets. *J. Biol. Chem.* 258: 7155–60

Assoian, R. K., Fleurdelys, B. E., Stevenson, H. C., Miller, P. J., Madtes, D. K., et al. 1987. Expression and secretion of type beta transforming growth factor by activated human macrophages. *Proc. Natl. Acad. Sci. USA* 84: 6020–24

Assoian, R. K., Sporn, M. B. 1986. Type-beta transforming growth factor in human platelets: release during platelet degranulation and action on vascular smooth muscle cells. *J. Cell Biol.* 102: 1712–33

Attardi, B., Keeping, H. S., Winters, S. J., Kotsuji, F., Maurer, R. A. Tren, P. 1989. Rapid and profound suppression of messenger ribonucleic acid encoding follicle-stimulating hormone β by inhibin from primate Sertoli cells. *Mol. Endocrinol.* 3: 280–87

Avallet, O., Vigier, M., Perrard-Sapori, M. H., Saez, J. M. 1987. Transforming growth factor β inhibits Leydig cell functions. *Biochem. Biophys. Res. Commun.* 146: 575–81

Barnard, J. A., Beauchamp, R. D., Coffey, R. J., Moses, H. L. 1989. Regulation of intestinal epithelial cell growth by transforming growth factor-beta. *Proc. Natl. Acad. Sci. USA* 86: 1578–82

Barton, D. E., Foellmer, B. E., Du, J., Tamm, J., Derynck, R., Franke, U. 1988. Chromosomal locations of TGF-β s 2 and 3 in the mouse and human. *Oncogene Res.* 3: 323031

Bassols, A., Massagué, J. 1988. Transforming growth factor-β regulates the expression and structure of extracellular matrix chondoitin/dermatan sulfate proteoglycans. *J. Biol. Chem.* 263: 3039–45

Behringer, R. R., Cate, R. L., Froelick, G. J., Palmiter, R. D., Brinster, R. L. 1990. Abnormal sexual development in transgenic mice chromically expressing Müllerian inhibitory substance. *Nature* 345: 167–70

Blanchard, M., Josso, N. 1974. Source of anti-Müllerian hormone synthesized by the fetal testis: Müllerian inhibiting activity of fetal bovine Sertoli cells in culture. *Pediatr. Res.* 8: 968–71

Boerner, P., Resnick, R. J., Racker, E. 1985. Stimulation of glycolysis and amino acid uptake in NRK-49F cells by transforming growth factor beta and epidermal growth factor. *Proc. Natl. Acad. Sci. USA* 82: 1350–53

Boyd, F. T., Massagué, J. 1989. Transforming growth factor-β inhibition of epithelial cell proliferation linked to the expression of a 53-kD membrane receptor. *J. Biol. Chem.* 264: 2272–78

Braun, T., Buschhausen-Denker, G., Bober, E., Tannich, E., Arnold, H. H. 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. *EMBO J.* 8: 701–9

Bryckaert, M. C., Lindroth, M., Lonn, A., Tobelem, G., Wasteson, A. 1988. Transforming growth factor (TGF-β) decreases the proliferation of human bone marrow fibroblasts by inhibiting the platelet-derived growth factor (PDGF) binding. *Exp. Cell Res.* 179: 311–21

Buchkovich, K., Duffy, L. A., Harlow, E. 1989. The retinoblastoma protein is phos-

phorylated during specific phases of the cell cycle. *Cell* 58: 1097–1105
Carr, B. I., Hayashi, I., Branum, E. L., Moses, H. L. 1986. Inhibition of DNA synthesis in rat hepatocytes by platelet-derived type β transforming growth factor. *Cancer Res.* 46: 2330–34
Cate, R. L., Mattaliano, R. J., Hession, C., Tizad, R., Farber, N. M., et al. 1986. Isolation of the bovine and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. *Cell* 45: 685–98
Centrella, M., McCarthy, T. L., Canalis, E. 1987. Transforming growth factor β is a bifunctional regulator of replication and collagen synthesis in osteoblast-enriched cell cultures from fetal rat bone. *J. Biol. Chem.* 262: 2869–74
Chambard, J.-C., Pouyssegur, J. 1988. TGF-β inhibits growth factor-induced DNA synthesis in hamster fibroblasts without affecting the early mitogenic events. *J. Cell Physiol.* 135: 101–7
Cheifetz, S., Andres, J. L., Massagué, J. 1988a. The transforming growth factor-β receptor type III is a membrane proteoglycan. Domain structure of the receptor. *J. Biol. Chem.* 263: 16984–91
Cheifetz, S., Bassols, A., Stanley, K., Ohta, M., Greenberger, J., Massagué, J. 1988b. Heterodimeric transforming growth factor-β. Biological properties and interaction with three types of cell surface receptors. *J. Biol. Chem.* 263: 10783–89
Cheifetz, S., Hernandez, H., Laiho, M., ten Dijke, P., et al. 1990. Determinants of cellular responsiveness to three transforming growth factor-β isoforms. Role of serum factors and distinct TGF-β receptor subsets. *J. Biol. Chem.* In press
Cheifetz, S., Like, B., Massagué, J. 1986. Cellular distribution of type I and type II receptors for transforming growth factor-β. *J. Biol. Chem.* 261: 9972–78
Cheifetz, S., Ling, N., Guillemin, R., Massagué, J. 1988c. A surface component on GH_3 pituitary cells that recognizes TGF-β, activin and inhibin. *J. Biol. Chem.* 263: 16984–91
Cheifetz, S., Massagué, J. 1989. The TGF-β receptor proteoglycan. Cell surface expression and ligand binding in the absence of glycosaminoglycan chains. *J. Biol. Chem.* 264: 12025–28
Cheifetz, S., Weatherbee, J. A., Tsang, M. L. S., Anderson, J. K., Mole, J. E., et al. 1987. The transforming growth factor-β system, a complex pattern of cross-reactive ligands and receptors. *Cell* 48: 409–15
Chen, J.-K., Hoshi, H., McKeehan, W. L. 1987. Transforming growth factor-β specifically stimulates synthesis of proteoglycan in human arterial smooth muscle cells. *Proc. Natl. Acad. Sci. USA* 84: 5287–91
Chen, P.-L., Scully, P., Shew, J.-Y., Wang, J. Y. J., Lee, W.-H. 1989. Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. *Cell* 58: 1193–98
Chiang, C. P., Nilsen-Hamilton, M. 1986. Opposite and selective effects of epidermal growth factor-β on the production of secreted proteins by murine 3T3 cells and human fibroblasts. *J. Biol. Chem.* 261: 10478–81
Chiquet-Ehrismann, R., Kalla, P., Pearson, C. A., Beck, K., Chiquet, M. 1988. Tenascin interferes with fibronectin action. *Cell* 53: 383–90
Cochet, C., Feige, J.-J., Chambaz, E. M. 1988. Bovine adrenocortical cells exhibit high affinity transforming growth factor-β receptors which are regulated by adrenocorticotropin. *J. Biol. Chem.* 263: 5707–13
Coffey, R. J., Kost, L. J., Lyons, R. M., Moses, H. L., LaRusso, N. F. 1987. Hepatic processing of transforming growth factor-β in the rat. *J. Clin. Invest.* 80: 750–57
Coffey, R. J. Jr., Bascom, C. C., Sipes, N. J., Graves-Deal, R., Weissman, B. E., Moses, H. L. 1988. Selective inhibition of growth-related gene expression in murine keratinocytes by transforming growth factor β. *Mol. Cell. Biol.* 8: 3088–93
Connor, T. B., Roberts, A. B., Sporn, M. B., Danielpour, D., Dart, L. L., et al. 1989. Correlation of fibrosis and transforming growth factor-β type 2 in the eye. *J. Clin. Invest.* 83: 1661–66
Daniel, T. O., Gibbs, V. C., Milfay, D. F., Williams, L. T. 1987. Agents that increase cAMP accumulation block endothelial c-*sis* induction by thrombin and transforming growth factor-β. *J. Biol. Chem.* 262: 11893–96
Davis, R. L., Weintraub, H., Lassar, A. B. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* 51: 987–1000
Dean, D. C., Newby, R. F., Bourgeosis, S. 1988. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor-β, and cAMP in human cell lines. *J. Cell Biol.* 106: 2159–70
DeCaprio, J. A., Ludlow, J. W., Lynch, D., Furukawa, Y., Griffin, J., et al. 1989. The product of the retinoblastoma susceptibility gene has properties of a cell regulatory element. *Cell* 58: 1085–95
de Martin, R., Haendler, B., Hofer-Warbinek, R., Gaugitsch, H., Wrann, M., et al. 1987. Complementary DNA for human

glioblastoma-derived T cell suppressor factor, a novel member of the transforming growth factor-β gene family. *EMBO J.* 6: 3673–77

Derynck, R., Jarrett, J. A., Chen, E. Y., Eaton, D. H., Bell, J. R., et al. 1985. Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. *Nature* 316: 701–5

Derynck, R., Jarrett, J. A., Chen, E. Y., Goeddel, D. V. 1986. The murine transforming growth factor-β precursor. *J. Biol. Chem.* 261: 4377–79

Derynck, R., Lindquist, P. B., Lee, A., Wen, D., Tamm, J., et al. 1988. A new type of transforming growth factor-β, TGF-β3. *EMBO J.* 7: 3737–43

Derynck, R., Rhee, L., Chen, E. Y., Van Tilburg, A. 1987. Intron-exon structure of human transforming growth factor-β precursor gene. *Nucleic Acids Res.* 15: 3188–89

Diaz, A., Varga, J., Jimenez, S. A. 1989. Transforming growth factor-β stimulation of lung fibroblast prostaglandin E_2 production. *J. Biol. Chem.* 264: 11554–11557

Dickinson, M. E., Kobrin, M. S., Silan, C. M., Kingsley, D. M., Justice, M. J., et al. 1990. Chromosomal localization of seven members of the murine TGF-β superfamily suggests close linkage to several morphogenetic mutant loci. *Genomics* In press

Dryja, T. P., Rapaport, J. M., Joyce, J. M., Petersen, R. A. 1986. Molecular detection of deletions involving band q14 of chromosome 13 in retinoblastomas. *Proc. Natl. Acad. Sci. USA* 83: 7391–94

Edwards, D. R., Murphy, G., Reynolds, J. J., Whitman, S. E., Docherty, A. J. P., et al. 1987. Transforming growth factor beta modulates the expression of collagenase and metalloproteinase inhibitor. *EMBO J.* 6: 1899–1904

Elingsworth, L. R., Brennan, J. E., Fok, K., Rosen, D. M., Bentz, H., et al. 1986. Antibodies to the N-terminal portion of cartilage-inducing factor A and transforming growth factor beta. *J. Biol. Chem.* 261: 12362–67

Espevik, T., Figari, I. S., Ranges, G. E., Palladino, M. A. 1988. Transforming growth factor-1 (TGF-β1) and recombinant tumor necrosis factor-alpha reciprocally regulate the generation of lymphokine-activated killer cell activity. *J. Immunol.* 140: 2312–16

Eto, Y., Tsuji, T., Takezawa, M., Takano, S., Yokogawa, Y., Shibai, H. 1987. Purification and characterization of erythroid differentiation factor (EGF) isolated from human leukemia cell line THP-1. *Biochem. Biophys. Res. Commun.* 142: 1095–1103

Falanga, V., Tiegs, S. L., Alstadt, S. P., Roberts, A. B., Sporn, M. B. 1987. Transforming growth factor-β: selective increase in glycosaminoglycan synthesis by cultures of fibroblasts from patients with progressive systemic sclerosis. *J. Invest. Dermatol.* 89: 100–4

Fanger, B. O., Wakefield, L. M., Sporn, M. B. 1986. Structure and properties of the cellular receptor for transforming growth-factor type-beta. *Biochemistry* 25: 3083–91

Feige, J.-J., Cochet, C., Rainey, W. E., Madani, C., Chambaz, E. M. 1987. Type β transforming growth factor affects adrenocortical cell-differentiated functions. *J. Biol. Chem.* 262: 13491–95

Fernandez-Pol, J. A., Talkad, V. D., Klos, D. J., Hamilton, P. D. 1987. Suppression of the EGF-dependent induction of c-*myc* proto-oncogene expression by transforming growth factor β in a human breast carcinoma cell line. *Biochem. Biophys. Res. Commun.* 144: 1197–1205

Florini, J. R., Roberts, A. B., Ewton, D. Z., Falen, S. L., Flanders, K. C., Sporn, M. B. 1986. Transforming growth factor-β. A very potent inhibitor of myoblast differentiation, identical to the differentiation inhibitor secreted by Buffalo rat liver cells. *J. Biol. Chem.* 261: 16509–13

Forage, R. G., Ring, J. M., Brown, P. W., McInerney, B. V., Cobon, G. S., et al. 1986. Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. *Proc. Natl. Acad. Sci. USA* 83: 3091–95

Frater-Schröder, M., Muller, G., Birchmeier, W., Bohlen, P. 1986. Transforming growth factor-β inhibits endothelial cell proliferation. *Biochem. Biophys. Res. Commun.* 137: 295–302

Friend, S. H., Bernards, R., Rogelj, S., Weinberg, R. A., Rapaport, J. M., et al. 1986. A human DNA segment with properties of the gene that predisposes to retinoblastoma and osteosarcoma. *Nature* 323: 643–46

Frolik, C. A., Dart, L. L., Meyers, C. A., Smith, D. M., Sporn, M. B. 1983. Purification and initial characterization of a type beta transforming growth factor from human placenta. *Proc. Natl. Acad. Sci. USA* 80: 3676–80

Frolik, C. A., Wakefield, L. M., Smith, D. M., Sporn, M. B. 1984. Characterization of a membrane receptor for transforming growth factor-β in normal rat kidney fibroblasts. *J. Biol. Chem.* 259: 10995–1000

Fujii, D., Brissenden, J. E., Derynck, R., Franke, U. 1986. Transforming growth

factor-β gene maps to human chromosome 19 long arm and to mouse chromosome 7. *Somat. Cell Mol. Genet.* 12: 281–88

Fung, Y.-K. T., Murphree, A. L., T'Ang, A., Qian, J., Hinrichs, S. H., Benedict, W. F. 1987. Structural evidence for the authenticity of the human retinoblastoma gene. *Science* 236: 1657–61

Gentry, L. E., Lioubin, M. N., Purchio, A. F., Marquardt, H. 1988. Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. *Mol. Cell Biol.* 8: 4162–68

Goodman, L. V., Majack, R. A. 1989. Vascular smooth muscle cells express distinct transforming growth factor-β receptor phenotypes as a function of cell density in culture. *J. Biol. Chem.* 264: 5241–44

Gray, A. M., Mason, A. V. 1990. Requirement for activin A and transforming growth factor-β1 pro-regions in homodimer assembly. *Science* 247: 1328–30

Graycar, J. L., Miller, D. A., Arrick, B. A., Lyons, R. M., Moses, H. L., Derynck, R. 1989. Human transforming growth factor-β3: recombinant expression, purification and biological activities in comparison with transforming growth factors β1 and β2. *Mol. Endocrinol.* 3: 1977–86

Green, H., Meuth, M. 1974. An established pre-adipose cell line and its differentiation in culture. *Cell* 3: 127–31

Gronwald, R. G. K., Seifert, R. A., Bowen-Pope, D. F. 1989. Differential regulation of expression of two platelet-derived growth factor-β. *J. Biol. Chem.* 264: 8120–25

Hanks, S. K., Armour, R., Baldwin, J. H., Maldonado, F., Spiess, J., Holley, R. W. 1988. Amino acid sequence of the BSC-1 cell growth inhibitor (polyergin) deduced from the nucleotide sequence of the cDNA. *Proc. Natl. Acad. Sci. USA* 85: 79–83

Heimark, R. L., Twardzik, D. R., Schwarz, S. M. 1986. Inhibition of endothelial cell regeneration by type-beta transforming growth factor from platelets. *Science* 233: 1078–1080

Heine, U. I., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Lam, H. Y. P., et al. 1987. Role of transforming growth factor-β in the development of the mouse embryo. *J. Cell Biol.* 105: 2861–76

Heino, J., Ignotz, R. A., Hemler, M. E., Crouse, C., Massagué, J. 1989. Regulation of cell adhesion receptors by transforming growth factor-β. Concomitant regulation of integrins that share a common β1 subunit. *J. Biol. Chem.* 264: 380–88

Heino, J., Massagué, J. 1990. Cell adhesion to collagen and decreased myogenic gene expression implicated in the control of myogenesis by TGF-β. *J. Biol. Chem.* 265: In press

Heino, J., Massagué, J. 1989. Transforming growth factor-β switches the pattern of integrins expressed in MG-63 human osteosarcoma cells and causes a selective loss of cell adhesion to laminin. *J. Biol. Chem.* 264: 21806–11

Hill, D. J., Strain, A. J., Elstow, S. F., Swenne, I., Milner, R. D. G. 1986. Bifunctional action of transforming growth factor-β on DNA synthesis in early passage human fetal fibroblasts. *J. Cell. Physiol.* 128: 322–28

Hino, M., Tojo, A., Miyazono, K., Miura, Y., Chiba, S., et al. 1989. Characterization of cellular receptors for erythroid differentiation factor on murine erythroleukemia cells. *J. Biol. Chem.* 264: 10309–14

Holley, R. W., Bohlen, P., Fava, R., Baldwin, J. H., Kleeman, G., Armour, R. 1980. Purification of kidney epithelial cell growth inhibitors. *Proc. Natl. Acad. Sci. USA* 77: 5989–92

Hotta, M., Baird, A. 1986. Differential effects of transforming growth factor type β on the growth and function of adrenocortical cells in vitro *Proc. Natl. Acad. Sci. USA* 83: 7795–99

Howe, P. H., Cunningham, M. R., Leof, E. B. 1990. Distinct pathways regulate transforming growth factor β1-stimulated proto-oncogene and extracellular matrix gene expression. *J. Cell. Physiol.* 142: 39–45

Howe, P. H., Leof, E. B. 1988. Transforming growth factor β1 treatment of AKR-2B cells is coupled through a pertussis-toxin-sensitive G-protein(s). *Biochem. J.* 261: 875–86

Hynes, R. O. 1987. Integrins: a family of cell surface receptors. *Cell* 48: 549–54

Ignotz, R. A., Endo, T., Massagué, J. 1987. Regulation of fibronectin and type-I collagen mRNA levels by transforming growth factor-β. *J. Biol. Chem.* 262: 6443–46

Ignotz, R. A., Heino, J., Massagué, J. 1989. Regulation of cell adhesion receptors by transforming growth factor-β. Regulation of vitronectin receptor and LFA-1. *J. Biol. Chem.* 264: 389–92

Ignotz, R. A., Massagué, J. 1985. Type β transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. *Proc. Natl. Acad. Sci. USA* 82: 8530–34

Ignotz, R. A., Massagué, J. 1986. Transforming growth factor-β stimulates the expression of fibronectin and collagen and

their incorporation into the extracellular matrix. *J. Biol. Chem.* 261: 4337–45
Ignotz, R. A., Massagué, J. 1987. Cell adhesion receptors as targets for transforming growth factor-β action. *Cell* 51: 189–97
Ignotz, R. A., Massagué, J. 1990. Regulation of phenotype by transforming growth factor-β: Role of extracellular matrix. In *Mechanisms of Differentiation*, ed. P. B. Fisher, Vol. 3. Boca Raton, Fla: CRC Press. In press
Inman, W. H., Colowick, S. P. 1985. Stimulation of glucose uptake by transforming growth factor-β: evidence for the requirement of epidermal growth factor receptor activation. *Proc. Natl. Acad. Sci. USA* 82: 1346–49
Ishibashi, T., Miller, S. L., Burstein, S. A. 1987. Type β transforming growth factor is a potent inhibitor of murine megakaryocytopoiesis in vitro. *Blood* 69: 1737–41
Jakowlew, S. B., Dillard, P. J., Kondiah, P., Sporn, M. B., Roberts, A. B. 1988a. Complementary deoxyribonucleic acid cloning of a novel transforming growth factor-β messenger ribonucleic acid from chick embryo chondrocytes. *Mol. Endocrinol.* 2: 747–55
Jakowlew, S. B., Dillard, P. J., Sporn, M. B., Roberts, A. B. 1988b. Complementary deoxyribonucleic acid cloning of a messenger ribonucleic acid encoding transforming growth factor beta 4 from chicken embryo chondrocytes. *Mol. Endocrinol.* 2: 1186–95
Jakowlew, S. B., Dillard, P. J., Sporn, M. B., Roberts, A. B. 1988c. Nucleotide sequence of chicken transforming growth factor-β1 (TGF-β1). *Nucleic Acids Res.* 16: 8730
Jennings, J. C., Mohan, S., Linkhart, T. A., Widstorm, R., Baylink, D. J. 1988. Comparison of the biological activities of TGF-β1 and TGF-β2: differential activity in endothelial cells. *J. Cell Physiol.* 137: 167–72
Jetten, A. M., Shirley, J. E., Stoner, G. 1986. Regulation of proliferation and differentiation of respiratory tract epithelial by TGF-β. *Exp. Cell Res.* 167: 539–49
Kanzaki, T., Olofsson, A., Morén, A., Wernstedt, C., Hellman, U., et al. 1990. TGF-β1 binding protein: A component of the large latent complex of TGF-β1 with multiple repeat sequences. *Cell* 61: 1051–61
Kehrl, J. H., Roberts, A. B., Wakefield, L. M., Jakowlew, S., Sporn, M. B., Fauci, A. S. 1986a. Transforming growth factor-β is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* 137: 3855–60
Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Jakowlew, S. B., Alvarez-Mon, M., et al. 1986b. Production of transforming growth factor-beta by human T lymphocytes and its potential role in the regulation of T cell growth. *J. Exp. Med.* 163: 1037–50
Keller, J. R., Mantel, C., Sing, G. K., Ellingsworth, L. R., Rusecetti, S. K., Ruscetti, F. W. 1988. Transforming growth factor-β1 selectively regulates early murine hematopoietic progenitors and inhibits the growth of IL-3 dependent myeloid leukemia cell lines. *J. Exp. Med.* 168: 737–50
Kerr, L. D., Miller, D. B., Matrisian, L. M. 1990. TGF-β1 inhibition of transin/stromelysin gene expression is mediated through a *fos*-binding sequence. *Cell* 61: 267–78
Kerr, L. D., Olashaw, N. E., Matrisian, L. M. 1988. Transforming growth factor β1 and cAMP inhibit transcription of epidermal growth factor- and oncogene-induced transin RNA. *J. Biol. Chem.* 263: 16999–7005
Kim, S.-J., Angel, P., Lafyatis, R., Hattori, K., Kim, K. Y., et al. 1990. Autoinduction of TGF-β1 is mediated by the AP-1 complex. *Mol. Cell. Biol.* 10: 1492–97
Kim, S.-J., Glick, A., Sporn, M. B., Roberts, A. B. 1989a. Characterization of the promoter region of the human transforming growth factor-β1 gene. *J. Biol. Chem.* 264: 402–8
Kim, S.-J., Jeang, K. T., Glick, A., Sporn, M. B., Roberts, A. B. 1989b. Promoter sequences of the human transforming growth factor-β1 gene responsive to transforming growth factor-β1 autoinduction. *J. Biol. Chem.* 264: 7041–45
Kimchi, A., Wang, X.-F., Weinberg, R. A., Cheifetz, S., Massagué, J. 1988. Absence of TGF-β receptors and growth inhibitory responses in retinoblastoma cells. *Science* 240: 196–98
Kimelman, D., Kirschner, M. 1987. Synergistic induction of mesoderm by FGF and TGF-β and the identification of an mRNA coding for EGF in the early *Xenopus* embryo. *Cell* 51:869–77
Knabbe, C., Lippman, M. E., Wakefield, L. M., Flanders, K. C., Kasid, A., et al. 1987. Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* 48: 417–28
Kondaiah, P., Sands, M. J., Smith, J. M., Fields, A., Roberts, A. B., et al. 1990. Identification of a novel transforming growth factor-β (TGF-β5) mRNA in *Xenopus laevis*. *J. Biol. Chem.* 265: 1089–93
Kovacina, K. S., Steele-Perkins, G., Purchio, A. F., Lioubin, M., Miyazono, K., et al. 1989. Interactions of recombinant and

platelet transforming growth factor-β1 precursor with the insulin-like growth factor/mannose 6-phosphate receptor. *Biochem. Biophys. Res. Commun.* In press

Kurokowa, M., Lynch, K., Podolsky, D. K. 1987. Effects of growth factors on an intestinal epithelial cell line: TGF-β inhibits proliferation and stimulates differentiation. *Biochem. Biophys. Res. Commun.* 142: 775–82

Laiho, M., De Caprio, J. A., Ludlow, J. W., Livingston, D. M., Massagué, J. 1990. Growth inhibition by TGF-β linked to suppression of retinoblastoma protein phosphorylation. *Cell* 62: 175–85

Laiho, M., Saksela, O., Andreasen, P. A., Keski-Oja, J. 1986. Enhanced production and extracellular deposition of the endothelial-type plasminogen activator inhibitor in cultured human lung fibroblasts by transforming growth factor-β. *J. Cell Biol.* 103: 2403–10

Laiho, M., Saksela, O., Keski-Oja, J. 1987. Transforming growth factor-β induction of type-1 plasminogen activator inhibitor. Pericellular deposition and sensitivity to exogenous urokinase. *J. Biol. Chem.* 262: 17467–74

Lassar, A. B., Buskin, J. N., Lockshon, D., Davis, R. L., Apone, S., et al. 1989a. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. *Cell* 58: 823–31

Lassar, A. B., Thayer, M. J., Overell, R. W., Weintraub, H. 1989b. Transformation by activated *ras* or *fos* prevents myogenesis by inhibiting expression of MyoD1. *Cell* 58: 659–67

Lawrence, D., Pircher, R., Jullien, P. 1985. Conversion of a high molecular weight latent beta-TGF from chicken embryo fibroblasts into a low molecular weight active beta-TGF under acidic conditions. *Biochem. Biophys. Res. Commun.* 133: 1026–34

Lee, W.-H., Shew, J.-Y., Hong, F. D., Sery, T. W., Donoso, L. A., et al. 1987. Human retinoblastoma susceptibility gene: cloning, identification, and sequence. *Science* 235: 1394–99

Lehnert, S. A., Akhurst, R. J. 1988. Embryonic expression pattern of TGF-β type 1 RNA suggests both paracrine and autocrine mechanisms of action. *Development* 104: 263–73

Leof, E. B., Proper, J. A., Goustin, A. S., Shipley, G. D., DiCorleto, P. E., Moses, H. L. 1986. Induction of c-*sis* mRNA and activity similar to platelet-derived growth factor by transforming growth factor β: a proposed model for indirect mitogenesis involving autocrine activity. *Proc. Natl. Acad. Sci. USA* 83: 2453–57

Li, L., Hu, J.-H., Olson, E. N. 1990. Different members of the *jun* proto-oncogene family exhibit different patterns of expression in response to type β transforming growth factor. *J. Biol. Chem.* 265: 1556–62

Like, B., Massagué, J. 1986. The antiproliferative effect of type β transforming growth factor occurs at a level distal from receptors for growth-activating factors. *J. Biol. Chem.* 261: 13426–29

Lin, P., Liu, C., Tsao, M.-S., Grisham, J. W. 1987. Inhibition of proliferation of cultured rat liver epithelial cells at specific cell cycle stages by transforming growth factor-β. *Biochem. Biophys. Res. Commun.* 143: 26–30

Lin, T., Blaisdell, J., Haskell, J. F. 1987. Transforming growth factor-β inhibits Leydig-cell steroidogenesis in primary culture. *Biochem. Biophys. Res. Commun.* 146: 387–94

Ling, N., Ying, S. Y., Ueno, N., Esch, F., Denoroy, L., Guillemin, R. 1985. Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. *Proc. Natl. Acad. Sci. USA* 82: 7217–21

Ling, N., Ying, S. Y., Ueno, N., Shimasaki, S., Esch, F., et al. 1986. Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. *Nature* 321: 779–82

Ludlow, J. W., DeCaprio, J. A., Huang, C.-M., Lee, W.-H., Paucha, E., Livingston, D. M. 1989. SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. *Cell* 56: 57–65

Ludlow, J. W., Shon, J., Pipas, J. M., Livingston, D. M., DeCaprio, J. A. (1990). The retinoblastoma susceptibility gene product undergoes cell cycle-dependent dephosphorylation binding to and release from SV40 large T-antigen. *Cell* 60: 387–96

Lund, L. R., Riccio, A., Andreasen, P. A., Nielsen, L. S., Kristensen, P., et al. 1987. Transforming growth factor-β is a strong and fast acting positive regulator of the level of type-1 plasminogen activator mRNA in WI-38 human lung fibroblasts. *EMBO J.* 6: 1281–86

Luyten, F. P., Cunninghan, N. S., Ma, S., Muthukumaran, N., Hammonds, R. G., et al. 1989. Purification and partial amino acid sequence of osteogenin, a protein initiating bone differentiation. *J. Biol. Chem.* 264: 13377–80

Lyons, K., Graycar, J. L., Lee, A., Hashmi, S., Lindquist, P. B., et al. 1989. *Bgr-1*, a mammalian gene related to *Xenopus Vg-*

1, is a member of the transforming growth factor gene superfamily. *Proc. Natl. Acad. Sci. USA* 86: 4554–58

Lyons, R. M., Keski-Oja, J., Moses, H. L. 1988. Proteolytic activation of latent transforming growth factor-β from fibroblast-conditioned medium. *J. Cell Biol.* 106: 1659–65

Machida, C. M., Muldoon, L. L., Rodland, K. D., Magun, B. E. 1988. Transcriptional modulation of transin gene expression by epidermal growth factor and transforming growth factor beta. *Mol. Cell. Biol.* 8: 2479–83

Madisen, L., Webb, N. R., Rose, T. M., Marquardt, H., Ikeda, T., et al. 1988. Transforming growth factor-β2: cDNA cloning and sequence analysis. *DNA* 7: 1–8

Madri, J. A., Pratt, B. M., Tucker, A. 1988. Phenotypic modulation of endothelial cells by transforming growth factor-β depends upon the composition and organization of the extracellular matrix. *J. Cell Biol.* 106: 1375–84

Majack, R. A. 1987. Beta-type transforming growth factor specifies organizational behavior in vascular smooth muscle cell cultures. *J. Cell Biol.* 105: 465–71

Mansbridge, J. N., Hanawalt, P. C. 1988. Role of transforming growth factor-beta in the maturation of human epidermal keratinocytes. *J. Invest. Dermatol.* 90: 336–41

Mason, A. J., Hayflick, J. S., Ling, N., Esch, F., Ueno, N., et al. 1985. Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. *Nature* 318: 659–63

Massagué, J. 1984. Type β transforming growth factor from feline sarcoma virus-transformed rat cells. Isolation and biological properties. *J. Biol. Chem.* 259: 9756–61

Massagué, J. 1985. Subunit structure of a high-affinity receptor for type β-transforming growth factor. *J. Biol. Chem.* 260: 7059–66

Massagué, J. 1987. The TGF-β family of growth and differentiation factors. *Cell* 49: 437–38

Massagué, J., Boyd, F. T., Andres, J. L., Cheifetz, S. 1990. Mediators of TGF-β action: TGF-β receptors and TGF-β-binding proteoglycans. *Ann. NY Acad. Sci.* 593: 59–72

Massagué, J., Cheifetz, S., Endo, T., Nadal-Ginard, B. 1986. Type β transforming growth factor is an inhibitor of myogenic differentiation. *Proc. Natl. Acad. Sci. USA* 83: 8206–10

Massagué, J., Kelly, B. 1986. Internalization of transforming growth factor-β and its receptor in Balb/c 3T3 fibroblasts. *J. Cell. Physiol.* 128: 216–22

Massagué, J., Kelly, B., Mottola, C. 1985. Stimulation by insulin-like growth factors is required for cellular transformation by type beta transforming growth factor. *J. Biol. Chem.* 260: 4551–54

Massagué, J., Like, B. 1985. Cellular receptors for type beta transforming growth factor. *J. Biol. Chem.* 260: 2636–45

Masui, T., Wakefield, L. M., Lechner, J. F., LaVeck, M. A., Sporn, M. B., Harris, C. C. 1986. Type β transforming growth factor is the primary differentiation inducing serum factor for normal human bronchial epithelial cells. *Proc. Natl. Acad. Sci. USA* 83: 2438–42

Matrisian, L. M., Leroy, P., Ruhlmann, C., Gesnel, M.-C., Breathnach, R. 1986. Isolation of the oncogene and epidermal growth factor-induced transin gene: complex control in rat fibroblasts. *Mol. Cell. Biol.* 6: 1679–86

McMahon, J. B., Richards, W. L., del Campo, C. C., Song, M.-K., Thorgiersson, S. S. 1986. Differential effects of transforming growth factor-β on proliferation of normal and malignant rat liver epithelial cells in culture. *Cancer Res.* 46: 4665–71

Melton, D. A. 1987. Translocation of a localized maternal mRNA to the vegetal pole of *Xenopus* oocytes. *Nature* 328: 80–82

Menko, A. S., Boettiger, D. 1987. Occupation of the extracellular matrix receptor, integrin, is a control point for myogenic differentiation. *Cell* 51: 51–57

Meunier, H., Rivier, C., Evans, R. M., Vale, W. 1988. Gonadal and extragonadal expression of inhibin α, βA and βB subunits in various tissues predicts diverse functions. *Proc. Natl. Acad. Sci. USA* 85: 247–51

Miller, D. A., Lee, A., Matsui, Y., Chen, E. Y., Moses, H. L., Derynck, R. 1989b. Complementary DNA cloning of the murine transforming growth factor-β3 (TGF-β3) precursor and the comparative expression of TGF-β3 and TGF-β1 messenger RNA in murine embryos and adult tissues. *Mol. Endocrinol.* 3: 1926–34

Miller, D. A., Lee, A., Pelton, R. W., Chen, E. Y., Moses, H. L., Derynck, R. 1989a. Murine transforming growth factor-β2 cDNA sequence and expression in adult tissues and embryos. *Mol. Endocrinol.* 3: 1108–14

Miyazono, K., Heldin, C.-H. 1989. Interaction between TGF-β1 and carbohydrate structures in its precursor renders TGF-β1 latent. *Nature* 388: 158–60

Miyazono, K., Hellman, U., Wernstedt, C., Heldin, C.-H. 1988. Latent high molecular weight complex of transforming growth factor β1. *J. Biol. Chem.* 263: 6407–15

Morrone, G., Cortese, R., Sorrentino, V. 1989. Post-transcriptional control of negative acute phase genes by transforming growth factor-beta. *EMBO J.* 8: 3767–71

Moses, H. L., Branum, E. L., Proper, J. A., Robinson, R. A. 1981. Transforming growth factor production by chemically transformed cells. *Cancer Res.* 41: 2842–48

Moses, H. L., Tucker, R. F., Leof, E. B., Coffey, R. J. Jr., Halper, J., Shipley, G. D. 1985. Type β transforming growth factor is a growth stimulator and growth inhibitor. In *Cancer Cells*, ed. J. Feramisco, B. Ozanne, C. Stiles, 3: 65–75. New York. Cold Spring Harbor Press

Mulder, K. M., Levine, A. E., Hernandez, X., McKnight, M. K., Brattain, D. E., Brattain, M. G. 1988. Modulation of c-*myc* by transforming growth factor-β in colon carcinoma cells. *Biochem. Biophys. Res. Commun.* 150: 711–16

Muldoon, L. L., Rodland, K. D., Magun, B. E. 1988. Transforming growth factor β and epidermal growth factor alter calcium influx and phosphatidylinositol turnover in Rat-1 fibroblasts. *J. Biol. Chem.* 263: 18834–41

Müller, G., Behrens, J., Nussbaumer, U., Bohlen, P., Birchmeier, W. 1987. Inhibitory action of transforming growth factor-β on endothelial cells. *Proc. Natl. Acad. Sci. USA* 84: 5600–4

Murthy, U. S., Anzano, M. A., Stadel, J. M., Greig, R. 1988. Coupling of TGF-β induced mitogenesis to G-protein activation in AKR-2B cells. *Biochem. Biophys. Res. Commun.* 152: 1228–35

Mustoe, T. A., Pierce, G. F., Thomason, A., Gramates, P., Sporn, M. B., Deuel, T. F. 1987. Transforming growth factor beta induces accelerated healing of incisional wounds in rats. *Science* 237: 1333–36

Nadal-Ginard, B. 1978. Commitment, fusion, and biochemical differentiation of a myogenic cell line in the absence of DNA synthesis. *Cell* 15: 855–64

Nakamura, T., Takio, K., Eto, Y., Shibai, H., Titani, K., Sugino, H. 1990. Activin-binding protein from rat ovary is follistatin. *Science* 247: 836–38

Nakamura, T., Tomita, Y., Hirai, R., Yamaoka, K., Kaji, K., Ichihara, A. 1985. Inhibitory effect of transforming growth factor-β on DNA synthesis of adult rat hepatocytes in primary culture. *Biochem. Biophys. Res. Commun.* 133: 1042–50

Noda, M., Rodan, G. A. 1987. Type-β transforming growth factor (TGF-β) regulation of alkaline phosphatase expression and other phenotype related mRNAs in osteoblastic rat osteosarcoma cells. *J. Cell Physiol.* 133: 426–37

Noda, M., Yoon, K., Prince, C. W., Butler, W. T., Rodan, G. A. 198. Transcriptional regulation of osteopontin production in rat osteosarcoma cells by type β transforming growth factor. *J. Biol. Chem.* 263: 13916–21

Nugent, M. A., Newman, M. J. 1989. Inhibition of normal rat kidney cell growth by transforming growth factor-β is mediated by collagen. *J. Biol. Chem.* 264: 18060–67

O'Connor-McCourt, M. D., Wakefield, L. M. 1987. Latent transforming growth factor-β in serum. *J. Biol. Chem.* 262: 14090–99

Ohta, M., Greenberger, J. S., Anklesaria, P., Bassols, A., Massagué, J. 1987. Two forms of transforming growth factor-β distinguished by multipotential haematopoietic progenitor cells. *Nature* 329: 539–41

Olson, E. N., Sternberg, E., Hu, J. S., Spizz, G., Wilcox, C. 1986. Regulation of myogenic differentiation by type beta transforming growth factor. *J. Cell Biol.* 103: 1799–805

Ottmann, O. G., Pelus, L. M. 1988. Differential proliferative effects of transforming growth factor-β on human hematopoietic progenitor cells. *J. Immunol.* 140: 2661–65

Padgett, R. W., St. Johnston, R. D., Gelbart, W. M. 1987. A transcript from a *Drosophila* pattern gene predicts a protein homologous to the transforming growth factor-β family. *Nature* 325: 81–84

Pearson, C. A., Pearson, D., Shibahara, S., Hofsteenge, J., Chiquet-Ehrismann, R. 1988. Tenascin: cDNA cloning and induction by TGF-β. *EMBO J.* 7: 2677–81

Pelton, R. W., Nomura, S., Moses, H. L., Hogan, B. L. M. 1989. Expression of transforming growth factor β-2 RNA during murine embryogenesis. *Development* 106: 759–68

Penttinen, R. P., Koyayashi, S., Bornstein, P. 1988. Transforming growth factor-β increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. *Proc. Natl. Acad. Sci. USA* 85: 1105–8

Pepinski, R. B., Sinclair, L. K., Chow, E. P., Mattaliano, R. J., Manganaro, T. F., et al. 1988. Proteolytic processing of Müllerian inhibiting substance produces a transforming growth factor-β-like fragment. *J. Biol. Chem.* 263: 18961–64

Pertovaara, L., Sistonen, L., Bos, T. J., Vogt, P. K., Keski-Oja, J., Alitalo, K. 1989. Enhanced *jun* gene expression is an early genomic response to transforming growth

factor β stimulation. *Mol. Cell Biol.* 9: 1255–62

Petraglia, F., Vaughan, J., Vale, W. 1989. Inhibin and activin modulate the release of gonadotropin-releasing hormone, human chorionic gonadotropin, and progesterone from cultured human placenta cells. *Proc. Natl. Acad. Sci. USA* 86: 5114–17

Pfeilschifter, J., D'Sousa, S. M., Mundy, G. R. 1987. Effects of transforming growth factor-β on osteoblastic osteosarcoma cells. *Endocrinology* 121: 212–18

Pietenpol, J. A., Holt, J. T., Stein, R. W., Moses, H. L. 1990. Transforming growth factor-β1 suppression of *c-myc* gene transcription: Role in inhibition of keratinocyte proliferation. *Proc. Natl. Acad. Sci. USA* 87: 3578–62

Pinney, D. F., Pearson-White, S. H., Konieczny, S. F., Latham, K. E., Emerson, C. P. Jr. 1988. Myogenic lineage determination and differentiation: evidence for a regulatory gene pathway. *Cell* 53: 781–93

Pircher, R., Jullien, P., Lawrence, D. A. 1986. β-Transforming growth factor is stored in human blood platelets as a latent high molecular weight complex. *Biochem. Biophys. Res. Commun.* 136: 30–37

Postlethwaite, A. E., Keski-Oja, J., Moses, H. L., Kang, A. H. 1987. Stimulation of the chemotactic migration of human fibroblasts by transforming growth factor beta. *J. Exp. Med.* 165: 251–56

Purchio, A. F., Cooper, J. A., Brunner, A. M., Lioubin, M. N., Gentry, L. E., et al. 1988. Identification of mannose 6-phosphate in two asparagine-linked sugar chains on recombinant transforming growth factors-β1 precursor. *J. Biol. Chem.* 263: 14211–15

Raghow, R., Postlethwaite, A. E., Keski-Oja, J., Moses, H. L., Kang, A. H. 1987. Transforming growth factor-β increases steady state levels of type I procollagen and fibronectin messenger RNAs posttranscriptionally in cultured human dermal fibroblasts. *J. Clin. Invest.* 79: 1285–88

Rappolee, D. A., Brenner, C. A., Schultz, R., Mark, D., Werb, Z. 1988. Developmental expression of PDGF, TGF-alpha, and TGF-β genes in preimplantation mouse embryos. *Science* 242: 1823–25

Rapraeger, A. 1989. Transforming growth factor (type β) promotes the addition of chondroitin sulfate chains to the cell surface proteoglycan (syndecan) of mouse mammary epithelia. *J. Cell Biol.* 109: 2509–18

Reiss, M., Sartorelli, A. C. 1987. Regulation of growth and differentiation of human keratinocytes by type β transforming growth factor and epidermal growth factor. *Cancer Res.* 47: 6705–9

Ristow, H. J. 1986. BSC-1 growth inhibitor type β transforming growth factor is a strong inhibitor of thymocyte proliferation. *Proc. Natl. Acad. Sci. USA* 83: 5531–34

Rivier, C., Rivier, J., Vale, W. 1986. Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. *Nature* 234: 205–8

Rizzino, A. 1987. Appearance of high affinity receptors for type β transforming growth factor during differentiation of murine embryonal carcinoma cells. *Cancer Res.* 47: 4386–90

Roberts, A. B., Anzano, M. A., Lamb, L. C., Smith, J. M., Sporn, M. B. 1981. New class of transforming growth factors potentiated by epidermal growth factor. *Proc. Natl. Acad. Sci. USA* 78: 5339–43

Roberts, A. B., Anzano, M. A., Meyers, C. A., Wideman, J., Blacher, R., et al. 1983. Purification and properties of a type beta transforming growth factor from bovine kidney. *Biochemistry* 22: 5692–98

Roberts, A. B., Anzano, M. A., Wakefield, L. M., Roche, N. S., Stern, D. F., Sporn, M. B. 1985. Type-β transforming growth factor: A bifunctional regulator of cellular growth. *Proc. Natl. Acad. Sci. USA* 82: 119–23

Roberts, A. B., Rosa, F., Roche, N. S., Coligan, J. E., Garfield, M., et al. 1989. Isolation and characterization of TGF-β2 and TGF-β5 from medium conditioned by *Xenopus* XTC cells. *Growth Factors.* In press

Roberts, A. B., Sporn, M. B. 1990. The transforming growth factor-betas. In *Peptide Growth Factors and Their Receptors*, ed. M. Sporn, A. B. Roberts. Heidelberg: Springer-Verlag. In press

Roberts, A. B., Sporn, M. B., Assoian, R. K., Smith, J. M., Roche, N. S., et al. 1986. Transforming growth factor type β: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation in vitro. *Proc. Natl. Acad. Sci. USA* 83: 4167–71

Roberts, C. J., Birkenmeir, T. M., McQuillan, J. J., Akiyama, S. K., Yamada, S. S., et al. 1988. Transforming growth factor-β stimulates the expression of fibronectin and of both subunits of the human fibronectin receptor by cultured human lung fibroblasts. *J. Biol. Chem.* 263: 4586–92

Rook, A. H., Kehrl, J. H., Wakefield, L. M., Roberts, A. B., Sporn, M. B., et al. 1986. Effects of transforming growth factor β on the functions of natural killer cells: depressed cytolytic activity and blunting

of interferon responsiveness. *J. Immunol.* 136: 3916–20
Rosa, F., Roberts, A. B., Danielpour, D., Dart, L. L., Sporn, M. B., David, I. B. 1988. Mesoderm induction in amphibians: the role of TGF-β2-like factors. *Science* 236: 783–86
Rosen, D. M., Stempien, S. A., Thompson, A. Y., Seyedin, P. R. 1988. Transforming growth factor-beta modulates the expression of osteoblast and chondroblast phenotypes in vitro. *J. Cell. Physiol.* 134: 337–46
Rossi, P., Karsenty, G., Roberts, A. B., Roche, N. S., Sporn, M. B., de Crombrugghe, B. 1988. A nuclear factor 1 binding site mediates the transcriptional activation of a type I collagen promoter by transforming growth factor-β. *Cell* 52: 405–14
Ruoslahti, E., Pierschbacher, M. D. 1987. New perspectives in cell adhesion: RGS and integrins. *Science* 238: 491–95
Russell, W. E., Coffey, R. J., Ouellette, A. J., Moses, H. L. 1988. Transforming growth factor beta reversibly inhibits the early proliferative response to partial hepatectomy in the rat. *Proc. Natl. Acad. Sci. USA* 85: 5126–30
Sandberg, M., Vuorio, T., Hirvonen, H., Alitalo, K., Vuorio, E. 1988. Enhanced expression of TGF-β and c-*fos* mRNAs in the growth plates of developing human long bones. *Development* 102: 461–70
Sassone-Corsi, P., Sisson, J. C., Verma, I. 1988. Transcriptional autoregulation of the proto-oncogene *fos*. *Nature* 334: 314–19
Sato, Y., Rifkin, D. B. 1989. Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-β1-like molecule by plasmin during co-culture. *J. Cell Biol.* 109: 309–15
Schütte, J., Viallet, J., Nau, M., Segal, S., Fedorko, J., Minna, J. 1989. *jun-B* inhibits and c-*fos* stimulates the transforming and transactivating activities of c-*jun*. *Cell* 59: 987–97
Scotto, L., Vaduva, P. I., Wager, R. E., Assoian, R. K. 1990. Type β1 transforming growth factor gene expression. A corrected mRNA structure reveals a downstream phorbol ester responsive element in human cells. *J. Biol. Chem.* 265: 2203–8
Segarini, P. R., Roberts, A. B., Rosen, M. D., Seyedin, S. M. 1987. Membrane binding characteristics of two forms of transforming growth factor-β. *J. Biol. Chem.* 262: 14655–62
Segarini, P. R., Rosen, D. M., Seyedin, S. M. 1989. Binding of transforming growth factor-β to cell surface proteins varies with cell type. *Mol. Endocrinol.* 3: 261–72
Segarini, P. R., Seyedin, S. M. 1988. The high molecular weight receptor to transforming growth factor-β contains glycosaminoglycan chains. *J. Biol. Chem.* 263: 8366–70
Seyedin, S. M., Segarini, P. R., Rosen, D. M., Thompson, A. Y., Bentz, H., Graycar, J. 1987. Cartilage-inducing factor-B is a unique protein structurally and functionally related to transforming growth factor-β. *J. Biol. Chem.* 262: 1946–49
Seyedin, S. M., Thomas, T. C., Thompson, A. Y., Rosen, D. M., Piez, K. A. 1985. Purification and characterization of two cartilage-inducing factors from bovine demineralized bone. *Proc. Natl. Acad. Sci. USA* 82: 2267–71
Shipley, G. D., Pittelkow, M. R., Wille, J. J. Jr., Scott, R. E., Moses, H. L. 1986. Reversible inhibition of normal human prokeratinocyte proliferation by type β transforming growth factor-growth inhibitor in serum-free medium. *Cancer Res.* 46: 2068–71
Shipley, G. D., Tucker, R. F., Moses, H. L. 1985. Type β-transforming growth factor/growth inhibitor stimulates entry of monolayer cultures of AKR-2B cells into S-phase after prolonged prereplicative interval. *Proc. Natl. Acad. Sci. USA* 82: 4147–51
Silberstein, G. B., Daniel, C. W. 1987. Reversible inhibition of mammary gland growth by transforming growth factor-β. *Science* 237: 291–93
Smith, J. C., Price, B. M. J., Van Nimmen, K. V., Huylebroeck, D. 1990. Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. *Nature*. In press
Sorrentino, V., Bandyopadhyay, S. 1989. TGFβ inhibits G_o/S-phase transition in primary fibroblasts. Loss of response to the antigrowth effect of TGFβ is observed after immortalization. *Oncogene* 4: 569–74
Sparks, R. L., Scott, R. E. 1986. Transforming growth factor type β is a specific inhibitor of 3T3 T mesenchymal stem cell differentiation. *Exp. Cell Res.* 165: 345–52
Spencer, F. A., Hoffmann, F. M., Gelbart, W. M. 1982. Decapentaplegic: A gene complex affecting morphogenesis in *Drosophila melanogaster*. *Cell* 28: 451–61
Sporn, M. B., Roberts, A. B., Shull, J. H., Smith, J. M., Ward, J. M., Sodek, J. 1983. Polypeptide transforming growth factors isolated from bovine sources and used for wound healing in vivo. *Science* 219: 1329–31

Sporn, M. B., Roberts, A. B., Wakefield, L. M., de Crombrugghe, B. 1987. Some recent advances in the chemistry and biology of transforming growth factor-β. *J. Cell Biol.* 105: 1039–45

Takehara, K., LeRoy, E. C., Grotendorst, G. R. 1987. TGF-β inhibition of endothelial cell proliferation: Alteration of EGF binding and EGF-induced growth-regulatory (competence) gene expression. *Cell* 49: 415–22

Tashjian, A. H., Voelkel, E. F., Lazzaro, B., Singer, F. R., Roberts, A. B., et al. 1985. α and β human transforming growth factors stimulate prostaglandin production and bone resorption in cultured mouse calvaria. *Proc. Natl. Acad. Sci. USA* 82: 4535–38

ten Dijke, P., Hansen, P., Iwata, K. K., Pieler, C., Foulkes, J. G. 1988a. Identification of another member of the transforming growth factor type β gene family. *Proc. Natl. Acad. Sci. USA* 85: 4715–19

ten Dijke, P., Iwata, K. K., Thorikay, M., Schwedes, J., Stewart, A., Pieler, C. 1990. Molecular characterization of transforming growth factor type β3. *Ann. NY Acad. Sci.* In press

ten Dijke, P., van Kessel, A. H. M. G., Foulkes, J. G., Le Beau, M. M. 1988b. Transforming growth factor type-β 3 maps to human chromosome 14, region q23–q24. *Oncogene* 3: 721–24

Thompson, K. L., Assoian, R., Rosner, M. R. 1988. Transforming growth factor-β increases transcription of the genes encoding the epidermal growth factor receptor and fibronectin in normal rat kidney fibroblasts. *J. Biol. Chem.* 263: 19519–24

Thompson, N. L., Flanders, K. C., Smith, J. M., Ellingsworth, L. R., Roberts, A. B., Sporn, M. B. 1989. Expression of transforming growth factor-β1 in specific cells and tissues of adult and neonatal mice. *J. Cell Biol.* 108: 661–69

Torti, F. M., Torti, S. V., Larrick, J. W., Ringold, G. M. 1989. Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor beta. *J. Cell Biol.* 108: 1105–13

Tsunawaki, S., Sporn, M. B., Ding, A., Nathan, C. 1988. Deactivation of macrophages by transforming growth factor-β. *Nature* 334: 260–62

Tucker, R. F., Shipley, G. D., Moses, H. L., Holley, R. W. 1984. Growth inhibitor from BSC-1 cells is closely related to the platelet type β transforming growth factor. *Science* 226: 705–7

Vaija, T. B., Rhodes, S. J., Taparowsky, E. J., Konieczny, S. F. 1989. Fibroblast growth factor and transforming growth factor β repress transcription of the myogenic regulatory gene MyoD1. *Mol. Cell Biol.* 9: 3576–79

Vale, W., Rivier, J., Vaughan, J., McClintock, R., Corrigan, A., et al. 1986. Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. *Nature* 321: 776–79

Van Obberghen-Schilling, E., Kondaiah, P., Ludwig, R. L., Sporn, M. B., Baker, C. C. 1987. Complementary deoxyribonucleic acid cloning of bovine transforming growth factor-β1 *Mol. Endocrinol.* 1: 693–98

Van Obberghen-Schilling, E., Roche, N. S., Flanders, K. C., Sporn, M. B., Baker, C. C. 1988. Transforming growth factor-β1 positively regulates its own expression in normal and transformed cells. *J. Biol. Chem.* 263: 7741–46

Varga, J., Rosenbloom, J., Jimenez, S. A. 1987. Transforming growth factor-β. (TGF-β) causes a persistent increase in steady-state amounts of type I and type III collagen and fibronectin mRNAs in normal human dermal fibroblasts. *Biochem. J.* 247: 597–604

Vigier, B., Picard, J.-Y., Campargue, J., Forest, M. G., Heyman, Y., Josso, N. 1984. Production of anti-Müllerian hormone: another homology between Sertoli and granulosa cells. *Endocrinology* 114: 1315–20

Vigier, B., Forest, M. G., Eychenne, B., Bezard, J., Garrigou, O., et al. 1989. Anti-Müllerian hormone produces endocrine sex reversal of fetal ovaries. *Proc. Natl. Acad. Sci. USA* 86: 3684–88

Wahl, S. M., Hunt, D. A., Wakefield, L. M., McCartney-Francis, N., Wahl, L. M., et al. 1987. Transforming growth-factor beta (TGF-β) induces monocyte chemotaxis and growth factor production. *Proc. Natl. Acad. Sci. USA* 84: 5788–92

Wahl, S. M., Hunt, D. A., Wong, H. L., Dougherty, S., McCartney-Francis, N., et al. 1988. Transforming growth factor-β is a potent immunosuppressive agent that inhibits IL-1 dependent lymphocyte proliferation. *J. Immunol.* 140: 3026–32

Wakefield, L. M., Smith, D. M., Flanders, K. C., Sporn, M. B. 1988. Latent transforming growth factor-β from human platelets. *J. Biol. Chem.* 263: 7646–54

Wakefield, L. M., Smith, D. M., Masui, T., Harris, C. C., Sporn, M. B. 1987. Distribution and modulation of the cellular receptor for transforming growth factor-β. *J. Cell Biol.* 105: 965–75

Wang, E. A., Rosen, V., Cordes, P., Hewick, R. M., Kriz, M. J., et al. 1988. Purification and characterization of other distinct bone-inducing factors. *Proc. Natl. Acad. Sci. USA* 85: 9484–98

Wang, E. A., Rosen, V., D'Alessandro, J. S., Bauduy, M., Cordes, P., et al. 1990. Recombinant human bone morphogenetic protein induces bone formation. *Proc. Natl. Acad. Sci. USA* 87: 2220–24

Weeks, D. L., Melton, D. A. 1987. A maternal mRNA localized to the vegetal hemisphere in *Xenopus* eggs codes for growth factor related to TGF-β. *Cell* 51: 861–67

Weintraub, H., Tapscott, S. J., Davis, R. L., Thayer, M. J., Adam, M. A., et al. 1989. Activation of muscle-specific genes in pigment, nerve, fat, liver and fibroblasts cell lines by forced expression of MyoD. *Proc. Natl. Acad. Sci. USA* 86: 5434–38

Wilcox, J. N., Derynck, R. 1988. Developmental expression of transforming growth factors alpha and beta in mouse fetus. *Mol. Cell. Biol.* 8: 3415–22

Wozney, J. M. 1989. Bone morphogenetic proteins. *Prog. Growth Factor Res.* 1: 267–80

Wozney, J. M., Rosen, V., Celeste, A. J., Mistsock, L. M., Whitters, M. J., et al. 1988. Novel regulators of bone formation: molecular clones and activities. *Science* 242: 1528–34

Wrann, M., Bodmer, S., de Martin, R., Siepl, C., Hofer-Warbinek, R., et al. 1987. T Cell supressor factor from human glioblastoma cells is a 12.5 kd protein closely related to transforming growth factor-β. *EMBO J.* 6: 1633–36

Wright, W. E., Sassoon, D. A., Lin, V. K. 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. *Cell* 56: 607–17

Ying, S.-Y., Becker, A., Baird, A., Ling, N., Ueno, N., et al. 1986a. Type beta transforming factor (TGF-β) is a potent stimulator of the basal secretion of follicle stimulating hormone (FSH) in a pituitary monolayer system. *Biochem. Biophys. Res. Commun.* 135: 950–56

Ying, S.-Y., Becker, A., Ling, N., Ueno, N., Guillemin, R. 1986b. Inhibin and beta type transforming growth factor (TGF-β) have opposite modulating effects on the follicle stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. *Biochem. Biophys. Res. Commun.* 136: 969–75

Yu, J., Shao, L., Lemas, V., Yu, A. L., Vaughan, J., et al. 1987. Importance of FSH-releasing protein and inhibin in erythrodifferentiation. *Nature* 330: 765–67

EXHIBIT II

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TGF-β SIGNAL TRANSDUCTION

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KEY WORDS: TGF-β, receptor serine/threonine kinases, SMADs, growth factors, development, cancer

ABSTRACT

The transforming growth factor β (TGF-β) family of growth factors control the development and homeostasis of most tissues in metazoan organisms. Work over the past few years has led to the elucidation of a TGF-β signal transduction network. This network involves receptor serine/threonine kinases at the cell surface and their substrates, the SMAD proteins, which move into the nucleus, where they activate target gene transcription in association with DNA-binding partners. Distinct repertoires of receptors, SMAD proteins, and DNA-binding partners seemingly underlie, in a cell-specific manner, the multifunctional nature of TGF-β and related factors. Mutations in these pathways are the cause of various forms of human cancer and developmental disorders.

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CONTENTS

INTRODUCTION

The transforming growth factor β (TGF-β) family comprises a large number of structurally related polypeptide growth factors, each capable of regulating a fascinating array of cellular processes including cell proliferation, lineage determination, differentiation, motility, adhesion, and death. Expressed in complex temporal and tissue-specific patterns, TGF-β and related factors play a prominent role in the development, homeostasis, and repair of virtually all tissues in organisms, from fruitfly to human. Collectively, these factors account for a substantial portion of the intercellular signals governing cell fate.

TGF-β and related factors are multifunctional agonists whose effects depend on the state of responsiveness of the target cell as much as on the factors themselves. Given this multifunctional nature, it is not surprising, in retrospect, that the gradual discovery of these factors over the past 15 years has been made through very disparate lines of investigation. For example, the founding member of the family, TGF-β1, was identified as a regulator of mesenchymal growth and, separately, as an antimitogen in epithelial cells (see Table 1 for references). Activins were identified as endocrine regulators of pituitary function and, independently, as inducers of mesoderm in frogs. Bone morphogenetic proteins (BMPs) were identified as bone repair factors and, independently, as dorsalizing agents in *Drosophila*.

A listing of the current members of the TGF-β family and their most representative activities is presented in Table 1 along with citations of articles that review in depth the discovery and biology of these factors. Based on sequence comparisons between the bioactive domains, the TGF-β family can be ordered around a subfamily that includes mammalian BMP2 and BMP4 and their close homologue from *Drosophila*, Dpp. All other known family members progressively diverge from this group, starting with the BMP5 subfamily, followed by the GDF5

Table 1 The transforming growth factor β (TGF-β) family and representative activities[a]

Names [Homologues]	%	Representative activities (References)
BMP2 subfamily		
BMP2 [Dpp^D]	100	Gastrulation, neurogenesis, chondrogenesis, interdigital
BMP4	92	apoptosis; in frog: mesoderm patterning; in fly: dorsalization, eyes, wings. (1–3)
BMP5 subfamily		
BMP5 [60 A^D]	61	Along with BMPs 2 and 4, this subfamily participates in the
BMP6/Vgr1	61	development of nearly all organs; many roles
BMP7/OP1	60	in neurogenesis. (1, 2)
BMP8/OP2	55	
GDF5 subfamily		
GDF5/CDMP1	57	Chondrogenesis in developing limbs. (1, 4)
GDF6/CDMP2	54	
GDF7	57	
Vg1 subfamily		
GDF1 [$Vg1^X$]	42	Vg1: axial mesoderm induction in frog and fish. (4)
GDF3/Vgr2	53	
BMP3 subfamily		
BMP3/osteogenin	48	Osteogenic differentiation, endochondral bone formation,
GDF10	46	monocyte chemotaxis. (5)
Intermediate members		
Nodal [Xnr 1 to 3^X]	42	Axial mesoderm induction, left-right asymmetry. (1, 6)
Dorsalin	40	Regulation of cell differentiation within the neural tube. (7)
GDF8	41	Inhibition of skeletal muscle growth. (8)
GDF9	34	
Activin subfamily		
Activin βA	42	Pituitary follicle-stimulating hormone (FSH) production,
Activin βB	42	erythroid cell differentiation; in frog, mesoderm
Activin βC	37	induction. (3, 9, 10)
Activin βE	40	
TGF-β subfamily		
TGF-β1	35	Cell cycle arrest in epithelial and hematopoietic cells, control of
TGF-β2	34	mesenchymal cell proliferation and differentiation, wound
TGF-β3	36	healing, extracellular matrix production, immunosuppression. (11–14)
Distant members		
MIS/AMH	27	Müllerian duct regression. (15, 16)
Inhibin α	22	Inhibition of FSH production and other actions of activin. (9, 10)
GDNF	23	Dopaminergic neuron survival, kidney development. (17)

[a]All members listed have been identified in human and/or mouse. In *brackets*, important homologues from *Drosophila* (^{D}) and *Xenopus* (^{X}). %, percent of amino acid identity with human bone morphogenetic protein (BMP)2 over the mature polypeptide domain. GDF, growth and differentiation factor. CDMP, cartilage-derived morphogenetic protein. MIS/AMH, Müllerian inhibiting substance/anti-Müllerian hormone. GDNF, glial cell–derived neurotrophic factor.

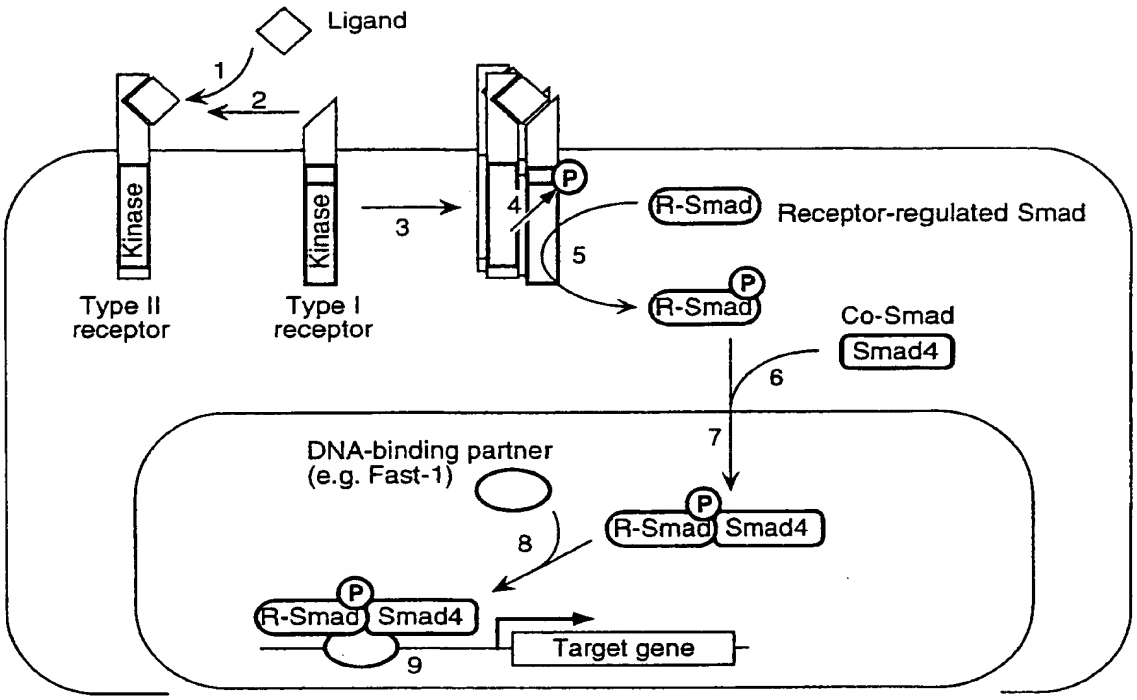


Figure 1 The transforming growth factor β (TGF-β)/SMAD pathway. Binding of a TGF-β family member to its type II receptor (*1*) in concert with a type I receptor (*2*) leads to formation of a receptor complex (*3*) and phosphorylation of the type I receptor (*4*). Thus activated, the type I receptor subsequently phosphorylates a receptor-regulated SMAD (R-Smad) (*5*), allowing this protein to associate with Smad4 (*6*) and move into the nucleus (*7*). In the nucleus, the SMAD complex associates with a DNA-binding partner, such as Fast-1 (*8*), and this complex binds to specific enhancers in targets genes (*9*), activating transcription.

(growth and differentiation factor 5) subfamily, the Vg1 subfamily, the BMP3 subfamily, various intermediate members, the activin subfamily, the TGF-β subfamily, and finally several distantly related members (Table 1) (1–17).

This review is devoted to a major accomplishment in this field over the past few years: the elucidation of a general mechanism by which TGF-β and related factors activate receptors at the cell surface and transduce signals to target genes (Figure 1). Some of these genes encode immediate effectors of ultimate cellular responses, such as cell cycle regulators that mediate antiproliferative responses or extracellular matrix components that determine cell adhesion, positioning, and movement. TGF-β and related factors regulate gene expression by bringing together two types of receptor serine/threonine protein kinases. One of these kinases phosphorylates the other, which in turn phosphorylates SMAD proteins. SMADs are a novel family of signal transducers that move into the nucleus and generate transcriptional complexes of specific DNA-binding ability. This review focuses on the structure and function of the TGF-β receptor family and

the SMAD family, their mechanisms of activation and regulation, and their disruption in human disease.

SIGNALING RECEPTORS

TGF-β and related factors signal through a family of transmembrane protein serine/threonine kinases referred to as the TGF-β receptor family. This family came to light with the cloning of an activin receptor (18), now referred to as ActR-II, with properties similar to those of TGF-β receptors identified in ligand cross-linking studies (19) and genetically implicated in TGF-β signal transduction (20). The cloning of ActR-II also revealed a striking similarity between this molecule and Daf-1, a previously identified orphan receptor from *Caenorhabditis elegans* (21). These findings provided the basis and impulse for the rapid identification of many other members of this receptor family.

Extensive evidence has accumulated to indicate that TGF-β family members signal through receptor serine/threonine kinases. One exception is the glial cell–derived neurotrophic factor (GDNF), which signals through the receptor tyrosine kinase Ret (17). GDNF was included in the TGF-β family because it has a set of cysteines that are characteristic of this family (22). However, GDNF is the most divergent family member and shows very little sequence similarity to other members (see Table 1). The next most divergent member, the Müllerian inhibiting substance (MIS; also known as anti-Müllerian hormone, AMH), signals through a TGF-β receptor family member, AMHR (23). GDNF therefore is in a class of its own aligned with the structurally diverse group of factors that signal through receptor tyrosine kinases.

Type I and II Receptor Families

Based on their structural and functional properties, the TGF-β receptor family is divided into two subfamilies: type I receptors and type II receptors (Figure 2). Type I receptors have a higher level of sequence similarity than type II receptors, particularly in the kinase domain. Vertebrate type I receptors form three groups whose members have similar kinase domains and signaling activities. In mammals, one group includes TβR-I, ActR-IB, and ALK7, another includes BMPR-IA and -IB, and the third includes ALK1 and ALK2.

As a result of being simultaneously cloned by different groups, most type I receptors have received different names. One practice has been to use the neutral nomenclature ALK (activin receptor–like kinase) and to adopt a more descriptive name when the physiological ligand becomes known. Thus, the TGF-β type I receptor originally known as ALK5 (24) is now called TβR-I (25). ActR-IB (previously also known as ALK4) (26) is an activin type I receptor (27), and BMPR-IA and -IB (previously known as ALK3 and ALK6, respectively) are

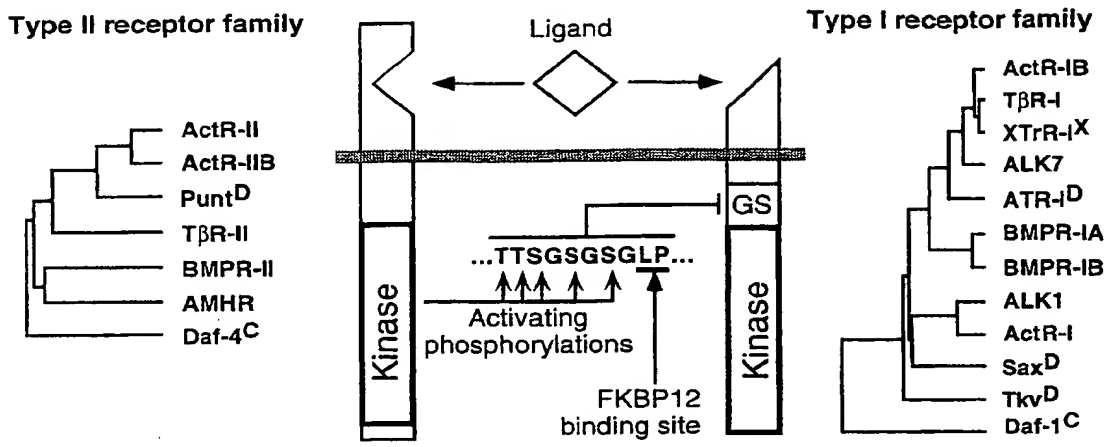


Figure 2 Type I and II TGF-β receptor families. In type I receptors, the protein kinase domain is preceded by the GS domain (GS). The characteristic GS sequence motif of TβR-I is shown, indicating the phosphorylation sites and the FKBP12-binding site. Listed members are from vertebrates unless otherwise indicated: D, *Drosophila*; C, *Caenorhabditis elegans*; X, *Xenopus*. The *dendrograms* indicate the relative level of amino acid sequence similarity in the kinase domain. Over this domain, ActR-II and Daf-4 have 40% sequence identity, and ActR- IB and Tkv have 60% identity.

BMP receptors (28, 29). Mammalian ALK7 (30, 31) and the related receptor XTrR-I from *Xenopus* (32) have no known ligand. ALK1 (also known as TSR-I) binds TGF-β (33) but does so more weakly than TβR-I (34) and is not known to mediate a TGF-β response (33). ALK2 is commonly referred to as ActR-I because it can bind activin and mediate certain activin responses in cultured cells (28, 33). However, the identity of its physiological ligand is a point of debate. ActR-I can also bind BMP2 and 4 (35, 36), and its mouse homologue can bind TGF-β when overexpressed (34, 37). Experiments using *Xenopus* embryo explants have shown that ActR-I/ALK2 mimics the mesoderm ventralizing activity of BMP4 but not the effects of activin or TGF-β, which suggests that ActR-I may function as a BMP receptor in vivo (39). Based on its expression pattern, it has been suggested that ALK2 may also function as an MIS/AMH type I receptor (38).

In vertebrates, the type II receptor subfamily includes TβR-II, BMPR-II, and AMHR, which selectively bind TGF-β (40), BMPs (36, 41, 42), and MIS (23, 43), respectively. ActR-II and -IIB bind activins when expressed alone or in concert with activin type I receptors (18, 44, 45). However, ActR-II and -IIB can bind BMPs 2, 4, and 7 and GDF5 in concert with BMP type I receptors (28, 46, 47).

Members of the TGF-β receptor family in invertebrates include Thick veins (Tkv) and Saxophone (Sax), which act as a Dpp type I receptors in *Drosophila* (48–51). Tkv most closely resembles the mammalian BMPR-I receptors,

whereas Sax is somewhat closer to mammalian ALK1 and ALK2. Punt acts as a Dpp type II receptor in concert with Tkv or Sax (52, 53). ATR-I is a *Drosophila* type I receptor closely related to mammalian TβR-I and ActR-IB (54). ATR-I can bind human activin, but its real ligand is unknown. In *C. elegans*, larval development is controlled by Daf-1 (21) and Daf-4 (55), which are thought to be type I and II receptors, respectively, for the BMP-like ligand Daf-7 (56).

Structural Features of the Receptors

THE EXTRACELLULAR DOMAIN Type I and II receptors are glycoproteins of approximately 55 kDa and 70 kDa, respectively, with core polypeptides of 500 to 570 amino acids including the signal sequence (18, 26, 40, 44, 57). The extracellular region is relatively short (approximately 150 amino acids), N-glycosylated (58, 59), and contains 10 or more cysteines that may determine the general fold of this region. Three of these cysteines form a characteristic cluster near the transmembrane sequence (54). The spacing of other cysteines varies and is more conserved in type I receptors than in type II receptors.

The transmembrane region and the cytoplasmic juxtamembrane region of type I and II receptors have no singular structural features. However, Ser213 in this region of TβR-II is phosphorylated by the receptor kinase in a ligand-independent manner and is required for signaling activity (60). Ser165 in the juxtamembrane region of TβR-I is phosphorylated by TβR-II in a ligand-dependent manner, and this appears to selectively modulate the intensity of different TGF-β responses (61).

THE GS DOMAIN A unique feature of type I receptors is a highly conserved 30–amino acid region immediately preceding the protein kinase domain (Figure 2). This region is called the GS domain because of a characteristic SGSGSG sequence it contains (62). Ligand-induced phosphorylation of the serines and threonines in the TTSGSGSG sequence of TβR-I by the type II receptor is required for activation of signaling (61–63), and the same happens with the activin type I receptor ActR-IB (64). Immediately following the SGSGSG sequence, all type I receptors have a Leu-Pro motif that serves as a binding site for the immunophilin FKBP12 (65, 66). FKBP12 may act as a negative regulator of the receptor signaling function. The penultimate residue in the GS domain, right at the boundary with the kinase domain, is always a threonine or a glutamine. As shown with TβR-I (63) and several other type I receptors (46, 64, 67–69), mutation of this residue to aspartate or glutamate endows the receptor with elevated kinase activity in vitro and constitutive signaling activity in the cell. Thus, the GS domain is a key regulatory region that may control the catalytic activity of the type I receptor kinase or its interaction with substrates.

THE KINASE DOMAIN The kinase domain in type I and II receptors conforms to the canonical sequence of a serine/threonine protein kinase domain (18, 24).

Consistent with this, type I receptors have been shown to phosphorylate their substrates—SMAD proteins—on serine residues (68, 70), whereas type II receptors phosphorylate themselves and type I receptors on serine and threonine residues but not tyrosine residues (40, 61–63, 71, 72). Autophosphorylation of TβR-II on tyrosine has been observed in vitro but not in vivo (73).

Conserved residues that in the crystal structure of other protein kinases coordinate ATP phosphate groups are essential for the activity of type I and II receptor kinases. These residues include a universally conserved β3-strand lysine (27, 74) and G217 in the glycine loop of TβR-I (75). The regulatory region known as the T loop in other protein kinases (76) contains two serines in TβR-II whose phosphorylation may enhance or inhibit the signaling activity of the receptor (60). A region of interest in the kinase domain of type I receptor kinases is the L45 loop that links two putative β strands. Replacement of the L45 loop in ActR-I with the L45 loop from TβR-I allows it to mediate TGF-β responses (77). Therefore this region may be involved in substrate recognition.

Type II receptors typically contain a very short C-terminal extension following the kinase domain, whereas type I receptors have essentially no C-terminal extension. Exceptions are the *C. elegans* receptor Daf-4 (55) and an alternative form of human BMPR-II (36, 41, 42, 78) that has long C-terminal extensions of unknown function. The C-terminal extension of TβR-II is phosphorylated (61), but its deletion does not impair signaling (79). This is in contrast to the important role that the C-terminal tail plays in signal transduction by tyrosine kinase receptors (80).

RECEPTOR VARIANTS Some members of the TGF-β receptor family exist in alternative forms. These forms arise from the presence or absence of the following: a 25–amino acid insert following the signal sequence in TβR-II (81, 82), a 61–amino acid insert in the same position in AMHR-II (23), two alternative N-terminal regions in Tkv (49, 50), two alternative extracellular juxtamembrane regions in ATR-I (54), small inserts in the extracellular and intracellular juxtamembrane regions of ActR-IIB (44), and a long C-terminal extension in BMPR-II (36, 41, 42, 78). The presence of the extracellular insert in ActR-IIB increases the affinity for activin (44). The functional significance of the other receptor variants is unknown.

LIGAND-RECEPTOR INTERACTIONS

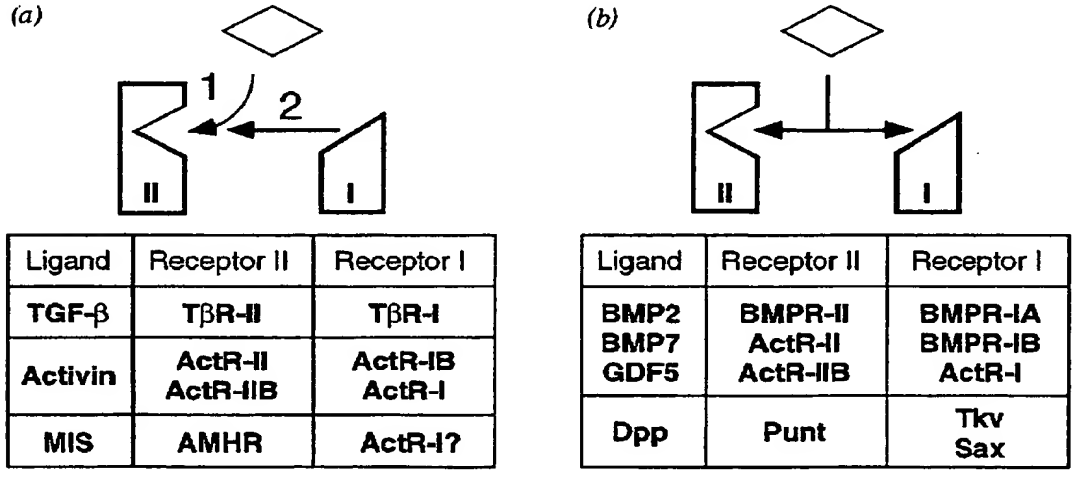
The Binding of Ligand to Signaling Receptors

LIGAND STRUCTURE: IMPLICATIONS FOR BINDING The bioactive forms of TGF-β and related factors are dimers held together by hydrophobic interactions and, in most cases, also by an intersubunit disulfide bond (83). Each monomer

contains three disulfide bonds interlocked into a tight structure known as the cystine knot (83). Insights into the possible regions of receptor contact are provided by the crystal structures of TGF-β2 (84, 85) and BMP7/OP-1 (86), the solution structure of TGF-β1 (87), and mutational analysis of TGF-β1 and TGF-β2 (88). The dimeric structure of these ligands suggests that they function by bringing together pairs of type I and II receptors, forming heterotetrameric receptor complexes. The pairing of receptors may be further specified by naturally occurring heterodimeric ligands such as TGF-β1.2 (19), TGF-β2.3 (89), and activin AB (90). The recombinant heterodimer BMP-4/7 is more potent in bioassays than BMP4 or BMP7 homodimers (91).

No species specificity has been described in the ligand-receptor interactions of the TGF-β system. Dpp receptors and Daf-4 can bind human BMPs (49, 50, 55), *dpp* phenotypes in flies can be rescued with a human *BMP4* transgene (92), and recombinant Dpp can induce endochondral bone formation in mammals (93).

TWO MODES OF BINDING TGF-β and related factors activate signaling by binding to and bringing together pairs of type I and II receptors. Two general modes of binding ligand have been observed (Figure 3). One mode involves direct binding to the type II receptor and subsequent interaction of this complex with the type I receptor, which, in effect, becomes recruited into the complex. This binding mode is characteristic of TGF-β and activin receptors. Type I receptors for these factors can recognize ligand that is bound to the type II



Ligand	Receptor II	Receptor I
TGF-β	TβR-II	TβR-I
Activin	ActR-II ActR-IIB	ActR-IB ActR-I
MIS	AMHR	ActR-I?

Ligand	Receptor II	Receptor I
BMP2 BMP7 GDF5	BMPR-II ActR-II ActR-IIB	BMPR-IA BMPR-IB ActR-I
Dpp	Punt	Tkv Sax

Figure 3 Two modes of ligand binding: (*a*) sequential binding, (*b*) cooperative binding. The ligands that bind according to each mode are listed together with the type I and II receptor combinations that they recognize. TGF, transforming growth factor; BMP, bone morphogenetic protein; GDF, growth and differentiation factor; MIS, Müllerian inhibiting substance.

receptors but not ligand that is free in solution (24, 33, 37, 72). This phenomenon was originally revealed by the receptor phenotype of TGF-β–resistant cell mutants (20, 94). TGF-β1 can bind to TβR-II in cell mutants lacking TβR-I but cannot bind to TβR-I in mutants lacking TβR-II. Restoration of TβR-II ligand-binding function, either by somatic fusion of a TβR-I defective cell with a TβR-II defective cell (95) or by transfection of a TβR-II cDNA (74), restores TGF-β binding to TβR-I. Furthermore, recombinant TβR-II binds TGF-β1 in solution (96–98). Like TβR-II, the type II receptors ActR-II (18), ActR-IIB (44, 45), and AMHR (23) also bind ligand when transfected in the absence of a type I receptor. Indeed, the original cloning of TβR-II (40) and ActR-II (18) was based on the ability of these receptors to bind ligand when overexpressed in COS cells.

The second binding mode is typical of BMP receptors and is cooperative, involving type I and II receptors that bind ligand with high affinity when expressed together but low affinity when expressed separately (36, 41, 42, 47) (Figure 3). Thus, BMPs 2, 4, and 7 and GDF5 bind weakly to the type II receptor BMPR-II expressed alone (36, 41, 42, 47) and to the type I receptors BMPR-IA or BMPR-IB expressed alone (29, 35) or in solution (99). ActR-II and -IIB are bona fide activin receptors that on their own bind BMP poorly if at all. However, ActR-II and -IIB can bind BMPs 2 and 7 in cooperation with BMPR-IA or BMPR-IB (28). This interaction mirrors what is observed with the Dpp receptor system. The Dpp type II receptor Punt, which is more closely related to ActR-II and -IIB than it is to BMPR-II, can recognize human activin (100). However, genetic evidence indicates that Punt acts as a Dpp receptor, and this evidence led to the finding that Punt binds Dpp or BMP poorly on its own but well in the presence of Tkv or Sax (52).

Accessory Receptors: Betaglycan and Endoglin

The original search for cell surface TGF-β–binding proteins using ligand cross-linking methods revealed the existence of binding proteins that were classified, according to their molecular weight, as type I and type II receptors (reviewed above) and type III receptors (19). Type III receptors detected by ligand cross-linking turned out to correspond to either one of two related proteins, betaglycan or endoglin (101–103). The evidence to date suggests that type III receptors do not have an intrinsic signaling function but regulate TGF-β access to the signaling receptors. There is no concrete evidence for type III receptors for other TGF-β family members.

BETAGLYCAN Betaglycan is a membrane-anchored proteoglycan (58, 104) with an 853–amino acid core protein (101, 102) that carries heparan sulfate and chondroitin sulfate glycosaminoglycan (GAG) chains attached to Ser535

and Ser546 (105, 106). In the cell, betaglycan appears to exist as noncolalent homodimers (107). GAG chains are not required for betaglycan to reach the cell surface or to bind TGF-β, as revealed by studies using cell mutants defective in GAG synthesis (108) and betaglycan mutants defective in GAG attachment sites (105, 106). However, GAG chains of betaglycan can bind fibroblast growth factor (109). The cytoplasmic region of betaglycan is short (43 amino acids) and lacks any discernible signaling motif (101, 102). This region is not required for the TGF-β binding and presentation functions of betaglycan (105), and its function remains unknown. The highest level of sequence similarity between betaglycan and endoglin is found in the cytoplasmic and transmembrane domains (110).

TGF-β binding activity has been demonstrated in separate N-terminal and C-terminal domains of the extracellular region of betaglycan (105, 111, 112). The N-terminal domain has sequence similarity to the corresponding region in endoglin (110). The C-terminal extracellular domain contains the GAG attachment sites (105) and shows sequence similarity to a region of the major urinary protein uromodulin, the pancreatic zymogen granule protein GP-2, and the sperm receptors Zp2 and Zp3 (113). The entire extracellular region of betaglycan may be shed into the medium (114), and it may act as a TGF-β antagonist, inhibiting binding to membrane receptors (105).

Betaglycan binds all three TGF-β forms with high affinity (115, 116) and facilitates TGF-β binding to the type II receptor (102, 117), forming a betaglycan/TGF-β/TβR-II complex in the process (117, 118). The role of betaglycan as a facilitator of TGF-β binding to the signaling receptors is most evident with TGF-β2. Like TGF-β1 and -β3, TGF-β2 signals through TβR-I and TβR-II (74, 95). However, unlike them, TGF-β2 has low intrinsic affinity for TβR-II (116) and is less potent than TGF-β1 in hematopoietic progenitor cells (119), myoblasts (117), and endothelial cells (116) that lack betaglycan. Transfection of betaglycan augments TGF-β2 binding and activity in these cells (117, 120). The ability of betaglycan to equalize the potency of all three TGF-β forms raises the possibility that betaglycan may not only concentrate TGF-β at the cell surface but may also stabilize TGF-βs in a conformation optimal for binding to the signaling receptors.

ENDOGLIN Endoglin is a cell surface molecule expressed at high levels in endothelial cells and at lower levels in monocytes, erythroid precursors, and other cell types (103, 121). Two splice variants of the cytoplasmic region give rise to human endoglin forms of 625 and 658 amino acids (122), each forming disulfide-linked dimers (103, 122). The sequence similarity between endoglin and betaglycan prompted an analysis of TGF-β binding to endoglin. This revealed that endoglin binds TGF-β1 and -β3, but unlike betaglycan, it does not

bind TGF-β2 (110). As with betaglycan, complexes between endoglin and TGF-β receptors have been observed (123).

However, the role of endoglin in TGF-β binding to signaling receptors is unclear. The TGF-β binding activity of endoglin is limited compared to that of betaglycan and is increased by coexpression of TβR-II. In fact, endoglin overexpression can diminish rather than enhance TGF-β responses in monocytes (121). As mentioned below, mutations in *endoglin* and *ALK1* give rise to similar human disorders (124–126). Endoglin and ALK1 therefore might act in the same pathway, with endoglin facilitating ligand binding to ALK1. Given the weak TGF-β–binding activities of both receptors, the common endoglin and ALK1 ligand may not have been identified yet.

Latent Ligands and Soluble Inhibitory Proteins

The activity of TGF-β and related factors is negatively regulated by various soluble proteins that prevent their interaction with membrane receptors (see Figure 6).

THE LATENT TGF-β COMPLEX Like all other members of its family, TGF-β is synthesized as the C-terminal domain of a precursor form that is cleaved before secretion from the cell (127, 128). However, the TGF-β propeptide, which is referred to as the latency associated peptide (LAP), remains noncovalently bound to TGF-β after secretion, retaining TGF-β in a latent form that cannot bind to betaglycan or the signaling receptors (129). Most cell types secrete TGF-β in this biologically inert form (12). Although LAP may be destroyed in the process of TGF-β activation, recombinant LAP retains TGF-β masking ability, and its injection in mice can inhibit endogenous TGF-β1 action (130).

A third component of the latent TGF-β complex is a large secretory glycoprotein known as latent TGF-β–binding protein (LTBP), which is disulfide-linked to LAP (131). LTBP is not required for the latency of the TGF-β complex but is implicated in the secretion, storage in the extracellular matrix, and eventual activation of this complex (131). LTBP comprises several forms generated from two genes and by alternative splicing: LTBP-1 in short and long forms (132) and LTBP-2 (133). Structurally, LTBPs contain a core of epidermal growth factor (EGF) repeats and eight-cysteine motifs organized in a fashion resembling fibrillin-1 and -2—two microfibrillar proteins whose mutations cause Marfan's syndrome and congenital contractural arachnodactyly, respectively (133). Like fibrillins, LTBP undergoes cross-linking by transglutaminases, forms fibrillar structures, and associates tightly with the extracellular matrix in mesenchymal and endothelial cells (134).

In tissue culture, LTBP associated with the extracellular matrix mediates storage of latent TGF-β and facilitates its activation (134, 135). Latent TGF-β

can be activated in vitro by acid, alkali, heat, limited proteolysis, or incubation by glycosidases (131). In tissue culture, activation of latent TGF-β may involve a combination of steps including the following: LAP proteolysis, binding to the mannose 6-phosphate/type II insulin-like growth factor receptor (Man6P/IGFR-II) via a mannose 6-phosphate group in LAP, cell-cell interactions between endothelial and vascular smooth-muscle cells, and binding to thrombospondin (131, 134–136). However, the physiological activation mechanism or mechanisms remain to be defined.

THE INHIBIN α CHAIN Inhibin is the name given to heterodimers between the inhibin α chain and an inhibin/activin β chain (137). Inhibin was identified as an inhibitor of follicle-stimulating hormone (FSH) production in pituitary cultures (9). The subsequent identification of activins as β-chain dimers with biological activities opposite those of inhibin led to the idea that inhibins and activins are mutual antagonists (9). Because inhibin can compete for binding to the activin receptors ActR-II and -IIB (18, 44), it might antagonize activin by binding to its receptors without triggering signaling, either by failing to recruit type I receptors or by failing to achieve their activation (138, 139). The inhibin α chain therefore can be regarded as an inhibitor that functions by associating with β chains generating activin receptor antagonists. However, some effects of inhibin could be mediated by as yet unidentified inhibin receptors.

THE ACTIVIN INHIBITOR FOLLISTATIN Follistatin is a soluble glycoprotein originally identified for its ability to inhibit pituitary FSH production (140) and later found to bind activin (141). Follistatin prevents activin binding to cell surface receptors (142). Paracrine as well as endocrine anti-activin effects of follistatin have been demonstrated in diverse tissues in mammals and *Xenopus* (140, 143–145). Follistatin can also bind to BMP-7, albeit with lower affinity than to activin (28), and may antagonize BMP signaling in vivo (145). Mammalian follistatin exists in forms of 288 and 315 amino acids generated by alternative splicing (146, 147). Follistatin is expressed in diverse mammalian tissues during development and in the adult (148–150) and in the Spemann's organizer in *Xenopus* embryos (145).

THE BMP INHIBITORS NOGGIN AND CHORDIN/SOG The Spemann's organizer, a signaling center at the dorsal lip of the *Xenopus* gastrula blastopore, secretes BMP antagonists—noggin and chordin—which allow neighboring cells to develop as neural or dorsal mesoderm rather than epidermal or ventral mesoderm tissues (151, 152). Although noggin and chordin are of unrelated primary structure, both bind BMP4 (but not TGF-β or activin), preventing its interaction with cell surface receptors (151, 152). Noggin, a 222–amino acid polypeptide that is secreted as a homodimer, was the first such antagonist to be identified

(153). In the mouse, a noggin homologue is expressed in specific regions of the nervous system (154). Chordin has four cysteine-rich repeats similar to those found in thrombospondin, α1 procollagen, and von Willebrand factor (155). In *Drosophila*, the short gastrulation gene product, Sog, is the structural and functional homologue of chordin (156–158) and prevents Dpp from signaling through its receptors (159). The structural differences between noggin and chordin may result in different abilities to diffuse from their source, interact with extracellular matrix, and/or recognize different members of the large and complex BMP subgroup.

MECHANISM OF RECEPTOR ACTIVATION

Studies on the mechanism of activation of serine/threonine kinase receptors have centered on TGF-β receptors. However, to the extent that these studies have been replicated with activin and BMP receptors, the same basic activation mechanism appears to operate in these receptors as well.

The Basal State

BASAL PHOSPHORYLATION The TGF-β type I receptor, TβR-I, is not phosphorylated in the basal state (62), but TβR-II, Act-R-II, and ActR-IIB are (40, 62, 64, 71, 160). Their basal phosphorylation is on serine residues and is partially retained in kinase-defective receptor mutants (62, 64). Some of the sites involved are in the C-terminal tail. Their functional significance is unclear: In one study, deletion of this entire region had no detectable effect on receptor signaling (79). Phosphorylation of other sites within TβR-II is dependent, directly or indirectly, on the activity of the receptor kinase (62, 64). In TβR-II, these sites include a serine in the juxtamembrane region and serines in the T-loop region of the kinase domain, and their phosphorylation modulates the signaling activity of TβR-II (60). What regulates the phosphorylation of these sites is not known.

BASAL RECEPTOR OLIGOMERIZATION The oligomeric state of endogenous TGF-β receptors is not known, but studies with transfected epitope-tagged receptors indicate that TβR-II can form ligand-independent homo-oligomers (107, 161). These complexes are thought to prime the formation of the heteromeric TβR-I/TβR-II receptor complex upon ligand binding.

Type I and II receptors have intrinsic affinity for each other, as manifested by the spontaneous association of TβR-I and TβR-II when overexpressed in insect cells or coincubated in vitro as recombinant proteins (96). In the absence of ligand, TβR-I and TβR-II (162) or ActR-IB and ActR-IIB (64) can form active complexes when overexpressed in mammalian cells. This interaction is mediated, at least in part, by the cytoplasmic regions because these

regions interact in a yeast two-hybrid system (36, 78, 96, 160). However, in transfected cells expressing moderate levels of TGF-β receptors (62) or activin receptors (138), the heteromeric receptor complex and, in particular, the phosphorylation and activation of the type I receptor are highly dependent on ligand binding.

FKBP12 BINDING The cytoplasmic domain of diverse type I receptors interacts with FKBP12 in yeast (36, 163, 164) and mammalian cells (66, 165, 166). FKBP12 is an abundant 12-kDa cytosolic protein with *cis-trans* peptidyl-prolyl isomerase (rotamase) activity (167). FKBP12 binds different proteins, some on its own and some as a target of various natural or synthetic immunosuppressants. On its own, FKBP12 binds to the ryanodine receptor and the inositol 1,4,5-triphosphate receptor, stabilizing the calcium channeling activity of these proteins (168, 169). In complex with the drug FK506, FKBP12 binds calcineurin, inhibiting calcineurin's phosphatase activity and thus its ability to activate the transcription factor NF-AT in the T-cell receptor signal transduction pathway (170). In complex with rapamycin, FKBP12 binds FRAP/RAFT, inhibiting its activity as a kinase in mitogenic signal transduction (171, 172).

FKBP12 binding to TβR-I inhibits TGF-β signaling (66, 166) by inhibiting TβR-I phosphorylation by TβR-II within the oligomeric receptor complex (66). FKBP12-receptor interaction is mediated by the active site of FKBP12 (66, 166) and a conserved Leu-Pro motif adjacent to the phosphorylation sites in the GS domain of the receptor (65, 66) (Figure 2). FKBP12 binds to the TGF-β type I receptor in the basal state and appears to be released upon TGF-β–induced formation of the receptor complex (66, 166). Mutant TβR-I receptors defective in FKBP12 binding have elevated basal signaling activity but normal signaling activity in the presence of ligand (66). Therefore, one function of FKBP12 may be to guard against spurious activation of TGF-β signaling by ligand-independent encounters of type I and II receptors.

OTHER RECEPTOR-BINDING PROTEINS TRIP-1 was identified as a TβR-II–interacting protein in a yeast two-hybrid screen (173). TRIP-1 contains several WD domains that may mediate protein-protein interactions, but the role of TRIP-1 is unknown. The interaction of TRIP-1 and TβR-II in mammalian cells is independent of ligand, requires the kinase activity of the receptor, and causes TRIP-1 phosphorylation (173).

The TβR-I cytoplasmic domain can interact with the farnesyl transferase-α subunit when both components are overexpressed in yeast or mammalian cells (164, 174, 175). It has been suggested that TGF-β may signal by regulating farnesyl transferase activity (174). However, this notion is controversial because the TGF-β receptor does not associate with the farnesyl transferase holoenzyme

(175). Furthermore, cells do not show a change in farnesyl transferase activity or in the farnesylation pattern of specific proteins in response to TGF-β (175).

The Activated State

RECEPTOR COMPLEX FORMATION Signals emanate from a TGF-β type I receptor when it is phosphorylated by its activator, the type II receptor. As first shown with TGF-β receptors (74), ligand binding induces the formation of a heteromeric complex of type I and II receptors (24, 25, 27, 33, 36, 41, 62, 64, 74, 138, 176) (Figure 1). Given the dimeric nature of the ligands, each monomer might contact one type I receptor and one type II receptor, thereby generating a heterotetrameric receptor complex. Indeed, that the ligand-induced heteromeric complex contains two or more type I receptor subunits and two or more type II receptor subunits is suggested by analysis of TGF-β receptor complexes on two-dimensional gel electrophoresis (25), coprecipitation of receptors containing distinct epitope tags (75), and genetic complementation between mutant type I receptors (75). The TGF-β receptor complex is extremely stable upon solubilization, resisting dissociation by ionic detergents and chaotropic agents (62). Formation of this complex is required for signaling. Using chimeric receptor constructs containing TβR-I and TβR-II kinase domains in different configurations, signaling is achieved only when type I and II receptor kinase domains are brought together (177–179).

TYPE II RECEPTOR KINASE ACTIVITY Ligand binding does not increase the overall phosphorylation of the type II receptors TβR-II, ActR-II, or ActR-IIB or their kinase activity in vitro (62, 64, 71, 162). Thus, type II receptors might be constitutively active kinases that require the ligand to interact with the type I receptor as a substrate. One caveat with this notion is that these studies have been done with moderately overexpressed receptors. It remains possible that type II receptors expressed at endogenous levels may undergo a ligand-induced increase in kinase activity. In any case, even when moderately overexpressed, type II receptors require ligand to phosphorylate their substrates, type I receptors.

TRANSPHOSPHORYLATION Formation of the ligand-induced receptor complex rapidly leads to phosphorylation of the type I receptor (Figure 1), as demonstrated with TGF-β (62, 162) and activin receptors (64, 180). This phosphorylation is catalyzed by the type II receptor, as shown by coexpression of wild-type and kinase-defective type I and II receptors in different combinations (62, 64, 162). TβR-I is phosphorylated by TβR-II at serine and threonine residues in the sequence TTSGSGSGLP of the GS domain (61–63) (Figure 2), and similar sites are phosphorylated in ActR-IB by activin type II receptors (64). In addition to these sites, TβR-II mediates phosphorylation of Ser165 in

the juxtamembrane region of TβR-I—a phosphorylation that may positively or negatively affect various TGF-β responses (61). TβR-I can catalyze its own phosphorylation in vitro, but there is no evidence that this occurs in vivo (63, 72, 75).

Signal Flow in the Receptor Complex

The events that transduce TGF-β signals start with type II receptor–mediated activation of the type I receptor. This receptor then phosphorylates and activates SMAD proteins, which carry the signal to the nucleus. This model is based on several lines of evidence. Mammalian cell mutants defective in either TβR-I (94) or TβR-II (20) lack a wide range of TGF-β responses. These responses are recovered in somatic hybrids between these two mutant phenotypes (95) or by transfection of the corresponding wild-type receptor (24, 72, 74). Work in *Drosophila* provides additional genetic evidence that Dpp signaling requires both type I and type II receptors (52, 53). Phosphorylation of serines and threonines in the GS domain of TβR-I is required for signaling (61–63). Alanine or valine mutations of any of these sites in TβR-I does not prevent phosphorylation of the other sites or receptor activation (63). However, mutation of three or more of these sites to alanine, valine, or acidic residues in TβR-I or ActR-IB prevents phosphorylation and signal transduction (63, 64, 180). Signaling is also inhibited when TβR-I phosphorylation is prevented by mutations in TβR-I or TβR-II that impair recognition of TβR-I as a substrate (75, 181), or by FKBP12 binding to the Leu-Pro motif in the GS domain (66).

A role of the type I receptor as the downstream signaling component in the receptor complex was originally inferred from the observation that the kinase activity of TβR-I is required for signal transduction and yet its substrate is neither TβR-I nor TβR-II (62). It was also shown that different type I receptors determine distinct responses to the same agonist (27, 182). Key evidence for a downstream role of the type I receptor was provided by the fact that hyperactive forms of TβR-I (63), ActR-IB (64), BMPR-IA and -IB (46, 68, 69), and Tkv (46, 67), generated by a mutation in the GS domain, have constitutive signaling activity in vivo. Signaling by hyperactive TβR-I also has been demonstrated in TβR-II–defective cells (63). The ability of purified BMP type I receptor to directly phosphorylate the activation sites of Smad1 in vitro (68) provides compelling evidence that in TGF-β receptor complexes, the signal flows from the type II receptor to the type I receptor and on to SMADs.

It is not clear whether activation of the type I receptor is based on an increase in its kinase activity, the appearance of substrate binding sites, or a combination of these two mechanisms. The hyperactive form TβR-I(T204D) has higher autokinase activity in vitro (63), suggesting that receptor activation may involve an increase in intrinsic kinase activity. On the other hand, it has been shown that

TβR-I activation results in Smad2 binding to the receptor complex (70, 183), suggesting that receptor activation may result in the generation of substrate docking sites.

In theory, the type II receptor could also signal independently of the type I receptor by phosphorylating other, as yet unidentified, signal-transducing substrates. However, no TGF-β responses have been described in cells lacking type I receptors. Overexpression of dominant-negative TβR-II receptor constructs can eliminate all TGF-β responses tested (79, 184) or only part of the TGF-β responses tested (185), depending on the assay conditions. Responses requiring a low level of signaling activity may be triggered by a residual level of activity in cells expressing dominant-negative receptors.

SMAD PROTEINS

The proteins of the SMAD family are the first identified substrates of type I receptor kinases and play a central role in the transduction of receptor signals to target genes in the nucleus (Figure 1).

SMADs as Mediators of TGF-β Signaling

The founding member of the SMAD family is the product of the *Drosophila* gene *Mad* (*mothers against dpp*) (186). *Mad* was identified in a genetic screen for mutations that exacerbate the effect of weak *dpp* alleles (187), and its discovery led to the identification of many related genes in nematodes and vertebrates. Three *Mad* homologues were identified in *C. elegans* and called *sma-2*, *-3*, and *-4* because their mutation causes small body size (188). Shortly thereafter, many homologues were described in vertebrates and named SMADs (for SMA/MAD related). *DPC4* (for "deleted in pancreatic carcinoma locus 4"), a gene frequently mutated or deleted in pancreatic cancer (189), also referred to as *Smad4*, was one of the first reported human SMADs. Human, mouse, and/or frog Smads 1–8 were cloned by screening EST (expressed sequence tag) databases or cDNA libraries for Mad homologues (46, 183, 190–198). Smad2 was idependently identified in a cDNA expression cloning screen for inducers of mesoderm formation in *Xenopus* embryos (199). Smads 6 and 7 were identified as shear stress–induced genes in endothelial cells (200).

Initial evidence that SMADs function downstream of TGF-β receptors was provided by the ability of *Mad* mutations to inhibit signaling by a hyperactive Tkv receptor construct (46, 67). The most compelling evidence came from the observation that in response to TGF-β and related agonists, SMADs are phosphorylated (46, 183, 192–194, 201), accumulate in the nucleus (46, 191, 199), and become transcriptionally active (191). This body of evidence placed SMADs squarely downstream of TGF-β receptors.

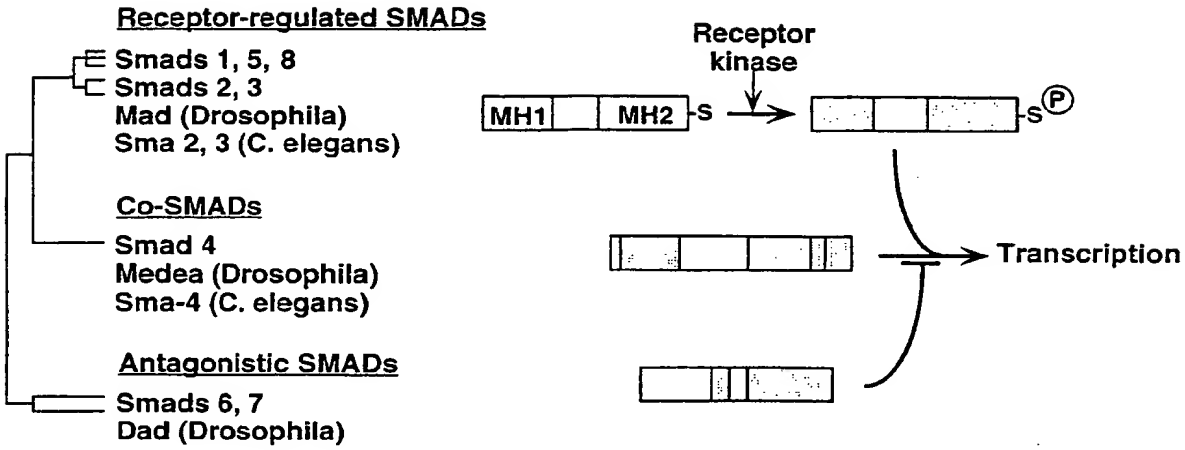


Figure 4 The SMAD family. Listed members are from vertebrates unless otherwise indicated. Vertebrate SMADs are highly conserved between human and *Xenopus*. The *dendrogram* indicates the relative level of amino acid sequence identity between vertebrate SMADs. The highly conserved MH1 and MH2 domains are indicated. Receptor-regulated SMADs are directly phosphorylated by TGF-β family type I receptors, and this phosphorylation allows association with a collaborating SMAD (co-SMAD). Antagonistic SMADs inhibit this SMAD activation process.

SMAD Subfamilies and Their Functions

Based on structural and functional considerations, SMADs fall into three subfamilies (Figure 4): (*a*) SMADs that are direct substrates of TGF-β family receptor kinases, (*b*) SMADs that participate in signaling by associating with these receptor-regulated SMADs, and (*c*) antagonistic SMADs that inhibit the signaling function of the other two groups.

Among the receptor-regulated SMADs, Smad1 and presumably its close homologues Smad5 and Smad8 are substrates for BMPR-I (68) and mediators of BMP signals (46, 190, 191, 202, 203, 203a). Smads 2 and 3 are TβR-I substrates (70, 183) and mediators of TGF-β and activin signals (190, 193, 195, 199, 201, 204). When overexpressed in *Xenopus* early embryos, Smad1 mimics the ability of BMP4 to ventralize mesoderm (190, 191, 202), whereas Smad2 mimics dorsal mesoderm induction and axis formation by activin (190, 199). In mammalian epithelial cells, Smads 2 and 3 mediate growth inhibition and transcriptional activation of TGF-β and activin reporter genes (183, 201). Mad and Sma's 2 and 3 also belong to this subfamily; they act as mediators of Dpp receptor signals (205) and Daf-4 signals (188), respectively.

Signaling by receptor-regulated SMADs requires the participation of a collaborating SMAD. The only known member of this group in vertebrates is Smad4. Smad4 associates with receptor-regulated SMADs when these become phosphorylated by the corresponding receptors (68, 183, 201, 206). Although Smad4 is similar to the receptor-regulated SMADs in overall structure,

it normally is not phosphorylated in response to agonists. Smad4 is required for Smad2- or Smad3-dependent growth inhibitory responses in mammalian cells, and a dominant-negative Smad4 construct interferes with Smad1 and Smad2 signaling in frog embryos and mammalian cells (183, 201). Smad4, therefore, participates in TGF-β, activin, and BMP signaling pathways as a shared partner of receptor-regulated SMADs. The *Medea* (206a–c) and *Sma-4* (188) gene products from *Drosophila* and nematode are close homologues of Smad4, and they may fulfill a similar function in these organisms.

Human Smads 6 and 7 and *Drosophila* Dad are a subfamily of structurally divergent SMADs whose only known activity is to inhibit the signaling function of receptor-activated SMADs. Smad6 preferentially inhibits BMP signaling (196, 207), Smad7 can inhibit TGF-β and BMP signaling (197, 208), and Dad inhibits Mad signaling (209). Additional SMADs have been identified in nematode, but their functional properties are complex, as inferred from genetic analysis (210).

Structural Features of SMADs

THE MH1 DOMAIN SMAD proteins contain highly conserved N-terminal and C-terminal domains (referred to as N and C domains, or MH1 and MH2 domains, respectively) and an intervening linker region that is of variable length and sequence (Figure 5). The MH1 domain has approximately 130 amino acids

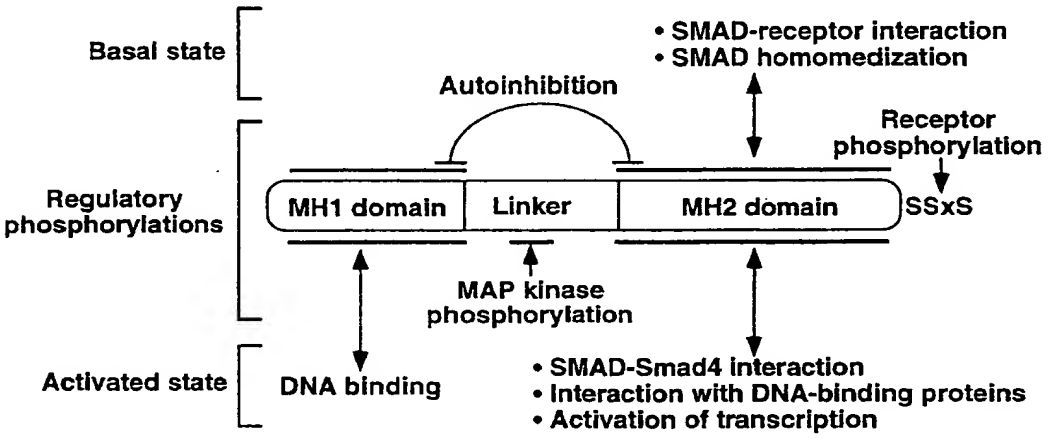


Figure 5 SMAD domains and their functions. In the basal state, SMADs form homo-oligomers and remain in an inactive state through an interaction between the MH1 and MH2 domains. Receptor-regulated SMADs interact with activated type I receptors via the MH2 domain and become activated by receptor-mediated phosphorylation at the C-terminal SS(V/M)S motif. In the activated state, SMADs associate with Smad4 and with DNA-binding proteins via the MH2 domain. The MH1 domain of some SMADs also participates in DNA binding, and the MH2 domain participates in transcriptional activation. MAP kinases phosphorylate some SMADs in the linker region, inhibiting SMAD accumulation in the nucleus.

and is highly conserved in receptor-regulated SMADs and Smad4 but not in inhibitory SMADs. In the basal state, the MH1 domain inhibits the transcriptional (191) and biological (199) activities of the MH2 domain. This inhibitory effect is likely due to the interaction between these two domains. Indeed, the MH1 domains of Smads 2 and 4 can physically interact with the respective MH2 domains, and overexpression of either MH1 domain as a separate protein can prevent the TGF-β–induced association of Smad2 and Smad4 MH2 domains (206).

The MH1 domain does not have a purely inhibitory function because it has DNA-binding activity in the activated state. The DNA-binding activity of the Mad MH1 domain is required for Dpp-induced activation of an enhancer within the *vestigial* wing-patterning gene (211). Likewise, the Smad4 MH1 domain contributes to the DNA-binding activity of a Smad2-Smad4 transcriptional complex (212). The DNA-binding activity of the Mad MH1 domain is inhibited by the presence of the MH2 domain (211), suggesting that the MH1 and MH2 domains may inhibit each other's function in the basal state. The contribution of the MH1 domains to the DNA-binding affinity and specificity of SMAD transcriptional complexes may vary depending on the particular target gene.

THE MH2 DOMAIN This domain contains receptor phosphorylation sites (in receptor-regulated SMADs) (68, 70), has effector function (191, 199), and is involved in several important protein-protein interactions (Figure 5). The canonical MH2 domain is about 200 amino acids long and contains a characteristic insert in the case of Smad4 and Sma-4 (183). Interactions between MH2 domains support the homo-oligomeric complexes that SMADs from all three subfamilies form in the basal state (201, 206, 207, 213, 214). The MH2 domains also mediate the association of receptor-regulated SMADs with type I receptors (70), with Smad4 upon receptor-mediated phosphorylation (206), and with DNA-binding factors (212, 215) (see below). The Smad2 MH2 domain is biologically active in frog mesoderm induction assays (199), and when fused to the DNA-binding domain of GAL4, the MH2 domains of Smad1 and Smad2 display agonist-independent transcriptional activity (191, 212). Smads 1 and 2 require the presence of the Smad4 MH2 domain to activate transcription (212). In the case of antagonistic SMADs, the MH2 domain is sufficient for their inhibitory effect (200, 207).

The crystal structure of the Smad4 MH2 domain has provided insights into the basis for some of these interactions (214). The Smad4 MH2 domain forms a homotrimer in the crystals, and Smad4 forms a trimer in solution. Each monomer consists of a β-sandwich core flanked by three α-helices in a bundle on one side and several loops and an α-helix on the other side. The trimer interfaces are formed by extensive contacts between the three-helix bundle of one monomer and the loops on the adjacent monomer. Tumor-derived mutations

in these interfaces destabilize and inactivate the homotrimer (see below). The trimer has the shape of a disc with the linker region emerging from one face. A loop referred to as the L3 loop protrudes from each monomer on the other face, and an α-helix referred to as helix-2 protrudes from each monomer on the edge of the disc. The L3 loops and the helix-2 may be sites for interaction with other proteins. Indeed, mutations in the L3 loop prevent Smad2 from interacting with the TGF-β receptor (217) and Smad4 from interacting with Smad2 (214). Based on sequence similarities, the overall structure of the MH2 domain is likely to be conserved in the other SMADs. Smads 6 and 7 lack the region corresponding to the third helix of the bundle, so they may form a different type of monomer-monomer interface (207).

THE LINKER REGION The linker region is highly variable in size and sequence. This region contributes to the formation of SMAD homo-oligomers (206, 213). In receptor-regulated SMADs, the linker region contains MAP-kinase phosphorylation sites (216). As discussed below, phosphorylation of these sites in response to MAP-kinase activation inhibits nuclear translocation of SMADs.

SIGNALING THROUGH SMADs

In the basal state, SMADs exist as homo-oligomers that reside in the cytoplasm (Figures 1 and 6). Upon ligand activation of the receptor complex, the type I receptor kinase phosphorylates specific SMADs, which then form a complex with Smad4 and move into the nucleus. In the nucleus, these complexes, either alone or in association with a DNA-binding subunit, activate target genes by binding to specific promoter elements.

SMADs as Receptor Substrates

PHOSPHORYLATION SITES SMADs are serine-phosphorylated in response to agonists, as shown with Smad1 in response to BMP2 or 4 (46, 68), Smad2 in response to TGF-β or activin (70, 193, 201), and Smad3 in response to TGF-β (183, 204). Although the kinetics of this phosphorylation are relatively slow ($t_{1/2} \sim 5$ min) when transfected SMADs are used, evidence shows that SMADs are direct substrates of the receptors. Smad1 is phosphorylated by highly purified, bacterially expressed BMPR-I kinase domain (68), Smad2 by immunoprecipitated TGF-β receptor complexes (70), and Smad3 by a TβR-I kinase preparation (183).

In vitro and in vivo, receptor-mediated phosphorylation occurs at serines in the C-terminal motif SS(V/M)S of Smad1 (68) or Smad2 (68, 70). This motif is also present in Smads 3, 5, and 8; *Drosophila* Mad; and *C. elegans* Smas-2 and -3. However, it is not present in the Smad4 subfamily or the inhibitory SMADs. This is consistent with the commonly observed lack of

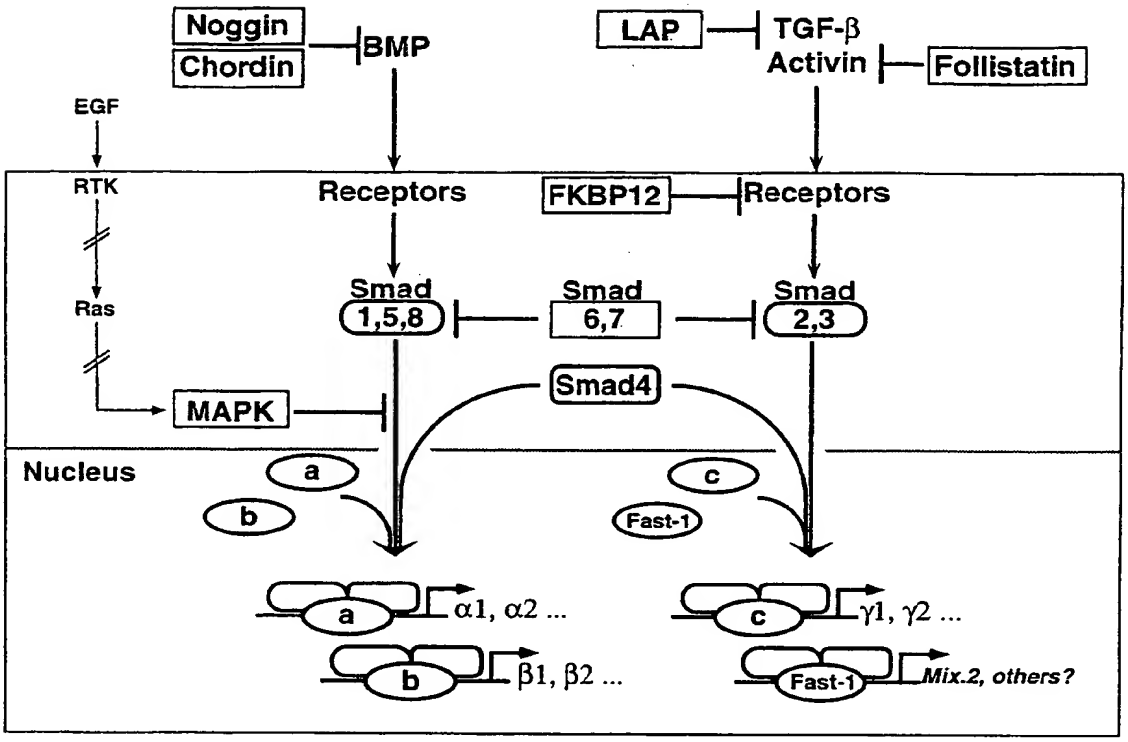


Figure 6 A model for generation of diverse gene responses by the SMAD system and their negative regulation. Smads 1, 5, and 8 are BMP receptor substrates and mediators of BMP gene response, whereas Smads 2 and 3 are substrates and mediators of TGF-β and activin receptors. Hypothetically each SMAD complex associates with different sequence-specific DNA-binding factors, of which Fast-1 is the first known example, and activates a distinct set of target genes. Negative regulation is provided by growth factor–sequestering proteins, FKBP12 binding to type I receptors, antagonistic SMADs, and SMAD phosphorylation by MAP kinases (in *gray boxes*). LAP, latency associated peptide.

agonist-induced phosphorylation in Smads 4, 6, and 7 (183, 196, 197, 201, 207, 208). Mutation of the serines in this sequence inhibits receptor-mediated phosphorylation of Smads 1 and 2 in vivo and in vitro and their association with Smad4 (68), accumulation in the nucleus (68, 70), interaction with DNA-binding proteins (212), and mediation of transcriptional responses (68, 70). Therefore, phosphorylation of this motif is required for SMAD activation.

SMAD-RECEPTOR ASSOCIATION Smad2 and Smad3 become transiently and selectively associated with the activated TGF-β receptor complex (70, 183, 217). This interaction is required for Smad2 phosphorylation because docking-defective Smad2 mutants are not phosphorylated in response to TGF-β (70, 217). The Smad2 phosphorylation sites themselves along with the adjacent sequence in the 11–amino acid C-tail region are not required for this interaction (217). In fact, phosphorylation of these sites appears to facilitate Smad2 dissociation from

the receptor, as either phosphorylation-defective Smad2 mutants or a kinase-defective TGF-β type I receptor mutant enhances SMAD-receptor association (70, 217). The transient nature of the SMAD-receptor interaction is consistent with the role of SMADs as carriers of receptor signals into the nucleus.

Mutational analysis of Smads 1 and 2 has identified their L3 loops as critical determinants of receptor docking interactions (217). The integrity of the L3 loop is necessary for interaction with the receptor and sufficient to dictate the specificity of this interaction. The L3-loop sequence is invariant among TGF-β–activated Smads (Smads 2 and 3) and BMP-activated Smads (Smads 1, 5, 9 and Mad) but differs at two positions between these two groups. Switching these two amino acids switches Smad1 and Smad2 activation by BMP and TGF-β, respectively. However, the isolated L3 loop is not sufficient to fully support this interaction. The SMAD-receptor interaction may require cooperativity provided by the oligomeric state of both the receptors and the SMADs.

Activated SMAD Complexes

Receptor-phosphorylated SMADs associate with Smad4, which functions as a shared partner required for transcriptional activation (Figure 6). Smad1 associates with Smad4 in response to BMPR-I activation (68, 201) and with Smads 2 and 3 in response to TβR-I or ActR-IB activation (201, 204). Smad4 can associate with these SMADs in yeast, which suggests that the interaction is direct (206). Based on structural considerations and the observation that mutations in the Smad4 L3 loop abolish the ability of Smad4 to associate with Smad2, the Smad4 L3 loop appears to mediate the association with receptor-activated SMADs (214). SMAD L3 loops, therefore, are implicated in two distinct types of interactions: (*a*) interaction with the receptors in the case of receptor-regulated SMADs and (*b*) interaction with receptor-activated SMADs in the case of Smad4. Functional interactions between receptor-regulated SMADs and a Smad4 family member may also occur in *Drosophila* (187) and *C. elegans* (188).

Nuclear Localization and Its Regulation

Nuclear translocation of receptor-activated SMADs occurs with kinetics that closely follow those of the agonist-induced phosphorylation and association with Smad4. Nuclear translocation of Smads 1 and 2 does not require Smad4, as determined using Smad4-defective cells (212). Smad4 is also translocated into the nucleus in response to TGF-β or BMP (204, 212), and this translocation requires the presence of Smad1 or Smad2 (212). Thus, it appears that receptor-activated SMADs bind Smad4 in the cytoplasm and carry it into the nucleus (212).

As central mediators of TGF-β family signals, SMADs are subject to different types of regulatory mechanisms that integrate and adapt their signaling

potential to the status of the cell. One mode of regulation is by phosphorylation of MAP-kinase sites in the linker region, inhibiting the accumulation of SMADs in the nucleus (216) (Figure 6). Agonists that activate Erk MAP kinases, such as epidermal growth factor (EGF) and hepatocyte growth factor, rapidly induce phosphorylation of Smad1 at serines in four PXSP motifs in the linker region. This phosphorylation is catalyzed by Erk MAP kinases and occurs independently of BMP receptor–mediated phosphorylation of Smad1. Erk-mediated phosphorylation inhibits nuclear accumulation of Smad1 without interfering with the association of Smad1 with Smad4. BMP responses that depend on nuclear accumulation of Smad1 are antagonized by activation of the Erk MAP-kinase pathway (216). This mechanism may underlie the ability of EGF to oppose osteogenic differentiation by BMP2 or the ability of fibroblast growth factor (FGF) to oppose the effect of BMP2 during limb bud outgrowth, digit formation, or tooth development (216). Other receptor-regulated SMADs also have potential MAP-kinase phosphorylation sites in their linker region. SMAD regulation by MAP kinases may therefore be a general phenomenon in the regulation of TGF-β signaling.

Transcriptional Complexes

The ability of SMADs to activate transcription was originally detected through the use of GAL4-Smad fusion constructs that activate GAL4 reporter gene (191). GAL4-Smad1 and GAL4-Smad2 constructs activate transcription in response to BMP4 and TGF-β, respectively, and their ability to do so requires Smad4, as determined using Smad4-defective cells (212). The first description of a natural SMAD transcriptional complex was made through studies on the activin response factor (ARF), a DNA-binding complex that forms in *Xenopus* embryo explants in response to activin or an endogenous factor, presumably Vg1 (218). ARF binds to a 50–base pair activin-response element (ARE) in the promoter of the homeobox gene *Mix.2*, an immediate-early activin response gene. The first component of ARF to be identified was the DNA-binding protein Fast-1, based on its ability to interact with a hexanucleotide repeat present in the activin-response element (219). Fast-1 is a novel member of the winged-helix family of putative transcription factors (also known as the HNF-3 family or the forkhead family) (220).

Fast-1 associates with Smad2 and Smad4, forming a ternary complex that binds to the ARE (212, 215) (Figure 6). Because Fast-1 is a nuclear protein (219), it probably binds to incoming Smad2-Smad4 complexes in the nucleus. The interaction involves a region within the C-terminal portion of Fast-1 and the MH2 domain of Smad2 (212, 215). Smad4 is not required for the Smad2-FAST1 interaction but contributes two essential functions to the resulting Smad2/Smad4/FAST-1 complex: Through its MH1 domain, Smad4

promotes binding of the complex to DNA, and through its MH2 domain, Smad4 activates transcription (212).

Other members of the winged-helix family might be DNA-binding partners of SMADs. However, members of structurally unrelated families might play this role as well. For example, the *Drosophila* gene *schnurri*, which encodes a zinc-finger protein with homology to various mammalian transcription factors, is genetically implicated in Dpp signaling (221, 222). Another Dpp-activated gene, *Ubx*, is activated via a cyclic AMP response element (CRE) adjacent to a sequence resembling a Mad-binding site (223). Paradoxically, mutation of this Mad-binding site did not interfere with Dpp activation of *Ubx*. SMADs may interact with certain target enhancers without the involvement of DNA-binding subunits (211, 223a,b), but the biological role of these interactions remains to be ascertained.

Response Elements

Numerous gene responses to TGF-β have been described, but only a fraction of these have the characteristics of an immediate transcriptional response. $p15^{Ink4b}$ and $p21^{Cip1}$ are cyclin-dependent kinase inhibitors whose rapid introduction in response to TGF-β mediates cell cycle arrest (224–227). Clusters of Sp1-like sites near the transcription start site of $p15^{Ink4b}$ and $p21^{Cip1}$ score as TGF-β–responsive regions in reporter gene assays (228, 229). TGF-β–stimulated expression of interstitial collagens and other extracellular matrix proteins underlies important roles of TGF-β in development and regenerative processes (11–13). The TGF-β–responsive regions of genes encoding such extracellular matrix proteins as collagen α1(I) (230), collagen α2(I) (231, 232), type 1 plasminogen activator inhibitor (PAI-1) (233, 234), elastin (235), and perlecan (236) resemble Sp1 sites or CTF/NF-I sites. However, some of these sequences also resemble the Mad-binding element of *vestigial* (211); thus they might be SMAD-binding sites. TGF-β and related factors can also cause rapid inhibition of gene transcription. Genes affected in this manner include *c-myc* (14) and the Cdk-activating phosphatase *cdc25A* (237); down-regulation of both genes by TGF-β mediates antiproliferative effects. Interestingly, transcriptional activation by TGF-β of PAI-1 (238), retinoic acid receptors (239), collagen α2(I), and other genes (238) appears to require AP-1 activity. Furthermore, a Fos-containing repressor has been implicated in the down-regulation of the secretory protease transin/stromalysin by TGF-β (240). Whether SMADs participate in all or even a majority of TGF-β gene responses is an open question.

Inhibition by Antagonistic SMADs

Vertebrate Smads 6 and 7 and *Drosophila* Dad are inhibitors of signaling by receptor-regulated SMADs (196, 197, 200, 207–209) (Figure 6). When overexpressed, Smad6 can inhibit BMP signaling and, partially, TGF-β signaling

(196), and Smad7 can inhibit TGF-β signaling (197, 208) and BMP signaling (197). At lower concentrations, however, Smad6 is a specific inhibitor of BMP signaling in frog embryos and mammalian cells (207). Dad inhibits Dpp signaling in *Drosophila* wing imaginal discs, and when introduced into frog embryos, Dad exhibits anti-BMP effects (209). Inhibitory SMADs participate in negative feedback loops that may regulate the intensity or duration of TGF-β responses. Thus, Smad7 expression is rapidly elevated in response to TGF-β (197), whereas Dad expression is elevated in response to Dpp (209). The expression of Smads 6 and 7 is elevated by shear stress in vascular endothelial cells (200), a reponse that might be mediated by autocrine TGF-β (241).

Inhibitory SMADs lack a C-terminal SSXS phosphorylation motif, and their N-terminal region has only short segments of MH1 domain homology (196, 197, 207–209). (Smad6 was originally reported as a truncated SMAD structure consisting of the MH2 domain only; see References 200, 242). One mechanism proposed to explain the inhibitory effects of Smads 6 and 7 is based on the observation that each of these SMADs can bind to diverse TGF-β family receptors and interfere with phosphorylation of receptor-regulated SMADs (196, 197, 208). This mechanism could account for the nonselective inhibition of BMP effects and TGF-β effects observed by overexpression of Smads 6 or 7. It is not known whether physiologic levels of inhibitory SMADs can interfere with receptor binding and phosphorylation of receptor-regulated SMADs.

A different mechanism may underlie the selective inhibition of BMP signaling by Smad6 (207). At low levels, Smad6 does not interfere with receptor-mediated phosphorylation of Smad1 but competes with Smad4 for binding to activated Smad1. In a yeast two-hybrid system, the Smad6 MH2 domain interacts with itself and with the Smad1 MH2 domain, but not with the MH2 domains of Smads 2 or 4. Smad6 binding to receptor-phosphorylated Smad1 yields a transcriptionally inert complex. Therefore, Smad6 appears to act as a Smad4 decoy for BMP-activated SMADs.

Other Kinases in TGF-β Signaling

Components of MAP-kinase cascades mediate numerous responses to mitogens, differentiation factors, inducers of apoptosis, radiation, and osmotic stress (243, 244). Several groups investigating whether TGF-β action affects the Erk subfamily of MAP kinases have reported activation (245), inhibition (246, 247), or no change (248) in the activity of these kinases after TGF-β treatment. A novel member of this family, TAK1 (TGF-β–activated kinase 1) was cloned based on its ability to activate a MAP kinase cascade in yeast (249). In mammalian cells, the activity of a transfected TAK1 is rapidly increased in response to TGF-β and BMP4 (249). Overexpression of a kinase-defective TAK1 mutant (249) or a truncated form of the TAK1 activator, TAB1 (250), diminishes the

TGF-β response of a reporter gene construct that contains an AP-1 site, implicating TAK1 in these responses. No effect of TAK1 on other TGF-β responses has been reported. TGF-β activation of the MAP-kinase JNK has been implicated in a similar transcriptional response and tentatively placed downstream of TAK1 (251, 252). However, the JNK-kinase response to TGF-β takes several hours, suggesting that JNK is not a primary transducer of TGF-β signals in these cells.

DISRUPTION OF TGF-β SIGNALING IN HUMAN DISORDERS

Alterations of TGF-β signaling pathways underlie many human disorders. A loss of growth inhibitory responses to TGF-β is often observed in cancer cells (253), and a gain of TGF-β activity is thought to play a central role in fibrotic disorders characterized by excessive accumulation of interstitial matrix material in the lung, kidney, liver, and other organs (254). Abnormal TGF-β activity is also implicated in inflammatory disorders (255–257). The phenotype of mice overexpressing or lacking specific TGF-β family members or their receptors has revealed that these alterations have profound effects on the development or homeostatis of many organs (1, 2, 4). However, direct evidence that disruption of TGF-β signaling is a cause of human disorders is provided by the following cases, in which genes encoding TGF-β family members, their receptors, or SMAD proteins are mutated (Figure 7).

TGF-β Receptor Mutations in Cancer

The effects of TGF-β on target cells include several forms of negative regulation of cell proliferation, such as induction of G1 arrest, promotion of terminal differentiation, or activation of cell death mechanisms (14, 258). Numerous reports have described deficiencies in these types of responses in human tumor–derived cell lines (253). Disruption of TGF-β signaling could therefore predispose or cause cancer.

This prediction was confirmed by the finding that the TGF-β type II receptor is inactivated by mutations in gastrointestinal cancers with microsatellite instability (259, 260). Microsatellite instabilty is common to many sporadic cancers and results from defects in DNA mismatch repair leading to nucleotide additions or deletions in simple repeated sequences—microsatellites—throughout the genome. The human *TβR-II* gene contains one such sequence, a 10-bp polyadenine repeat, starting at nucleotide 709 in the coding region of the extracellular domain. One- or two-base additions or deletions in this repeat occur in most sporadic colon cancers and gastric cancers with microsatellite instability, yielding truncated, inactive TβR-II products (260–262). Mutations in the *TβR-II* polyadenine repeat are also found in colon or gastric tumors from

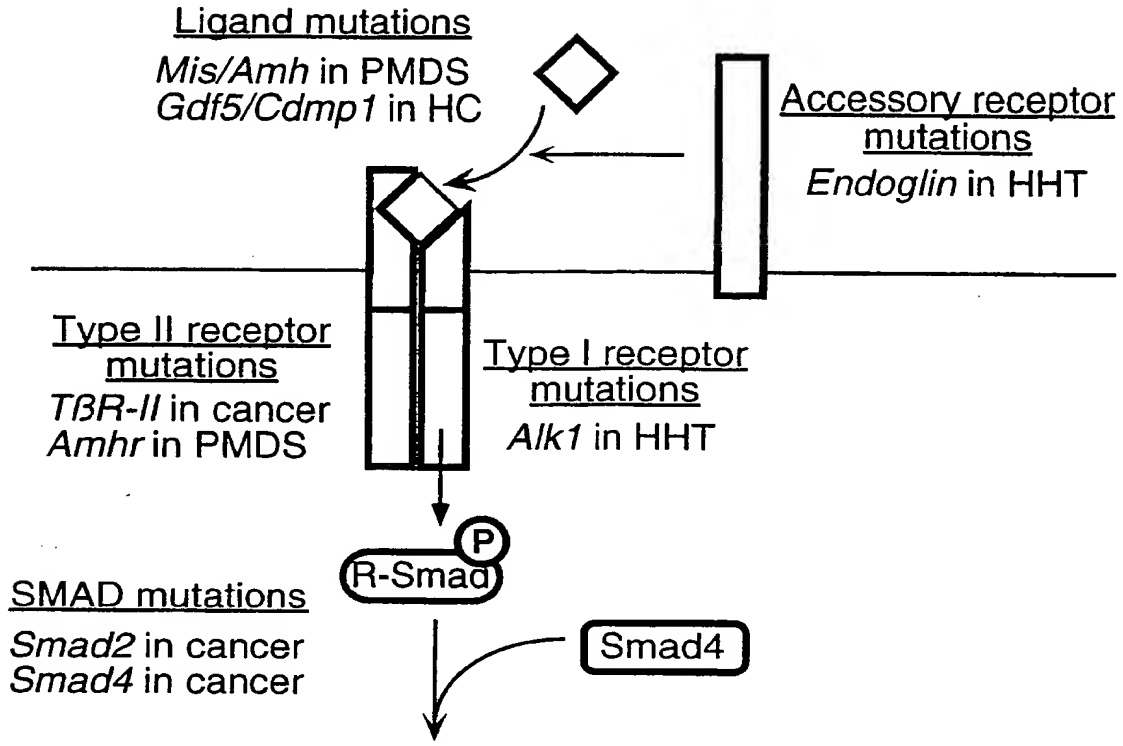


Figure 7 Inactivating mutations in TGF-β signal transduction genes in human disorders. *Mis/Amh* or *Amhr* is mutated in persistent Müllerian duct syndrome (PMDS); *Gdf5/Cdmp1* in hereditary chondrodysplasia (HC); *endoglin* or *Alk1* in hereditary hemorrhagic telangectasia (HHT); *TβR-II* in gastrointestinal cancers with somatic or hereditary microsatellite instability; *Smad2* in colon cancer; and *Smad4* in pancreatic, colon, and other cancers.

individuals with hereditary non-polyposis colon cancer (HNPCC) (263), a familial syndrome characterized by a high incidence of colon, endometrial, and gastric cancers. In most of these cases, both *TβR-II* alleles have mutations in the polyadenine repeat. In some cases, however, the second allele is inactivated by a different mutation, such as (*a*) an addition of a GT dinucleotide to a GT-GTGT sequence in the kinase domain coding region or (*b*) missense mutations, which are also predicted to inactivate this kinase (260, 261, 263). These results indicate that *TβR-II* shares the two-hit inactivation mechanism of other tumor suppressor genes.

Mutations in the *TβR-II* polyadenine repeat are rare in somatic or hereditary cancers of the endometrium, pancreas, liver, and breast (262, 264, 265) or in myelodisplastic syndrome (266) with microsatellite instability. This provides further evidence that mutations in the *TβR-II* polyadenine repeat are not just a random consequence of microsatellite instability but are specifically selected during the progression of colon and gastric cancers. Mutations elsewhere in

TβR-II have been described in T-cell lymphoma, gastric cancers, and head and neck carcinomas (267–269).

SMAD Mutations in Cancer

The TGF-β signaling network is also disrupted in cancer by mutations in *Smad2* and *Smad4/DPC4*. *Smad4/DPC4* was originally identified as a candidate tumor suppressor gene in chromosome 18q21 that was somatically deleted or mutated in half of all human pancreatic carcinomas (189). Biallelic *Smad4/DPC4* inactivation also occurs in a significant proportion of colorectal tumors (270, 271). *Smad4/DPC4* is infrequently mutated in breast (272), ovarian (272), head and neck (273), prostatic (271), esophageal, and gastric cancers (274). In the mouse, *Smad4* inactivation causes intestinal tumors in concert with inactivation of another tumor suppresor gene, *APC* (274a). *Smad2* is also located at 18q21, and it too is the target of inactivating mutations in colon cancer (193, 275, 276). Loss of TGF-β responsiveness in colon cancer therefore may be due to mutations in *TβR-II*, *Smad2*, or *Smad4/DPC4*. Interestingly, the preponderance of *Smad4/DPC4* mutations in pancreatic cancer, together with the low frequency of mutations in *TβR-II* in these tumors (264), raises the possibility that loss of Smad4 function may be selecting for resistance to an endogenous factor other than TGF-β itself.

Smad2 and *Smad4/DPC4* are inactivated in cancer by missense mutations, nonsense mutations, small deletions, frameshift mutations, or loss of the entire chromosomal region. Most of the missense mutations described fall in the MH2 domain (214), a region that is also the target of mutations in *Mad*, *sma-2*, and *sma-3* inactive alleles (186, 188). The location of these mutations is consistent with the effector role of the MH2 domain in SMAD signaling. Resolution of the crystal structure of the Smad4 MH2 domain has revealed that tumor-derived missense mutations in this domain often affect amino acids that are critical for monomer-monomer interactions within the Smad trimer (214). Such mutations weaken Smad homo-oligomerization and prevent TGF-β–induced Smad2-Smad4 association. Less frequently, tumor-derived mutations destabilize the folding of the MH2 domain (214). Tumor-derived missense mutations have also been identified in the MH1 domains of *Smad2* and *Smad4/DPC4*. These mutations inactivate SMAD function by increasing the affinity of the MH1 domain for the homologous MH2 domain, locking the molecule in an inhibited conformation (206). Several mutations from inactive alleles of *Mad* or *sma* genes map to the region corresponding to the L3 loop and are predicted to interfere with heteromeric Smad interactions (214) or Smad-receptor interactions (217). However, no such mutation has been described in human SMAD genes.

GDF5/CDMP1 Mutations in Hereditary Chondrodysplasia

The phenotypes of mice defective in specific members of the BMP and GDF subfamilies have indicated that despite their similar activities in tissue culture, each of these factors is rate limiting for a distinct subset of developmental processes, including the development of specific skeletal components (1, 2, 4). One example of this is provided by the finding that the *brachypodism* phenotype in mice is due to inactivating mutations in the *Gdf5* gene (277, 278). *Brachypodism* mice have numerous alterations in the length and number of bones in the limbs but retain a normal axial skeleton (277). This finding raised the possibility that the human GDF5 homologue, known as cartilage-derived morphogenetic protein 1 (CDMP1), might likewise be involved in skeletal abnormalities. This possibility was confirmed with the identification of a frameshift mutation in *Cdmp1* in individuals with the recessive chondrodysplasia syndrome, Hunter-Thompson type acromesomelic chondrodysplasia (279). The mutation found in this study is a 22-bp insertion in the mature region of CDMP1 and most likely yields an inactive product. The abnormalities in affected individuals are restricted to the limbs and are most severe in the distal bones, which are short and dislocated (279).

ALK1 and Endoglin Mutations in Hereditary Hemorrhagic Telangectasia

The accessory receptor endoglin (103) and the type I receptor ALK1 (33) are highly expressed in vascular endothelial cells. The genes encoding these products have been identified as the targets of inactivating mutations in human hereditary hemorrhagic telangectasia (124–126). This disorder is characterized by epithelial vascular dysplasia and a high propensity to hemorrhage in the nasal and gastrointestinal mucosa (280). The autosomal dominant nature of this disorder argues that maintenance of appropriate endoglin and ALK1 levels is crucial for vascular homeostasis. The similarity of the phenotypes caused by mutations in either gene suggests that both receptors function in a common pathway controlling the development of the vascular wall. Because endoglin and ALK1 are not effective at binding TGF-β (33, 34, 110), it is possible that these two receptors mediate the action of an as yet unidentified TGF-β family member in the vasculature.

MIS and MIS Receptor Mutations in Persistent Müllerian Duct Syndrome

During the development of the reproductive tract in mammals, the Müllerian duct gives rise to the uterus, fallopian tubes, and upper vagina (15, 16). Regression of the Müllerian duct in males is mediated by MIS/AMH from the

Sertoli cells of the fetal testis acting via its receptor, AMHR, on the mesenchymal cells adjacent to the Müllerian duct epithelium (23, 43, 281). Disruption of this process leads to the appearance of internal pseudohermaphroditism with uterine and oviductal tissues in affected males, a disorder known as persistence of Müllerian duct syndrome (PMDS) (282). PMDS has been shown to result from inactivating mutations in either *Mis/Amh* (283–285) or *Amhr* (285–287). A 27-bp deletion in *Amhr* is a common cause of PMDS (288). The phenotypes of mutations in *Mis/Amh* and *Amhr* are essentially the same, and they are copied in mice defective in the ligand, the receptor, or both (289). These observations suggest that unlike other TGF-β family members, MIS/AMH and its receptor have a highly specific and restricted role during development.

SUMMARY AND PROSPECTS

Recent progress has led to the elucidation of a general TGF-β signaling pathway in which the ligand causes the activation of a heteromeric protein kinase complex that subsequently phosphorylates a subset of SMAD proteins that move into the nucleus, where they activate specific target genes with the agency of DNA-binding partners. The cellular response to a TGF-β factor may be determined not only by the receptors and SMAD isoforms present in the cell but also by the available repertoire of DNA-binding partners. The response is further modulated by regulators of ligand binding, receptor activity, SMAD activation, or nuclear localization. All the central components of these pathways and many of their regulators are novel proteins of previously unknown function.

The combinatorial interactions that configure such TGF-β signaling pathways provide a basis for understanding the multifunctional nature of these factors. In principle, now it should be possible to determine which combination of receptors, SMAD proteins, and DNA-binding partners leads to each particular TGF-β gene response. This signaling process is based on a succession of discrete protein-protein and protein-DNA interactions. The structural elements that mediate each contact can now be investigated to ascertain how signaling specificity is enforced in the pathway. These protein interactions are of limited strength; thus they seem good candidates as drug targets. This prospect is interesting, for either gain or loss of TGF-β signaling processes underlies various developmental disorders, several forms of cancer, and other ailments in humans.

The progress made allows us to explain, in general terms, how a TGF-β signaling pathway works. However, what is described here will likely become, with time, only part of the explanation as the complexity of this pathway is exposed in full. We might yet learn that type II receptors phosphorylate a different set of transducers, or that type I receptors have other substrates besides SMAD proteins, or that SMADs have other functions besides activating

transcription. Furthermore, the recent emphasis on the transcriptional effects of TGFs and family members may have sidestepped other important responses to these factors; it is time to investigate these other responses as well. Clearly then, more work and more surprises lie ahead. However, the recent elucidation of the first contiguous TGF-β signaling pathway is a major milestone in this field and provides the framework for future research.

Literature Cited

1. Hogan BLM. 1996. *Genes Dev.* 10: 1580–94
2. Mehler MF, Mabie PC, Zhang DM, Kessler JA. 1997. *Trends Neurosci.* 20: 309–17
3. Harland RM. 1994. *Proc. Natl. Acad. Sci. USA* 91:10243–46
4. Kingsley DM. 1994. *Genes Dev.* 10:16–21
5. Cunningham NS, Paralkar V, Reddi AH. 1992. *Proc. Natl. Acad. Sci. USA* 89: 11740–44
6. Beddington R. 1996. *Nature* 381:116–17
7. Basler K, Edlund T, Jessell TM, Yamada T. 1993. *Cell* 73:687–702
8. McPherron AC, Lawler AM, Lee S-J. 1997. *Nature* 387:83–90
9. Vale W, Hsueh A, Rivier C, Yu J. 1990. See Ref. 290, pp. 211–48
10. Gaddy-Kurten D, Tsuchida K, Vale W. 1995. *Recent Prog. Horm. Res.* 50:109–29
11. Massagué J. 1990. *Annu. Rev. Cell. Biol.* 6:597–641
12. Roberts AB, Sporn MB. 1990. See Ref. 290, pp. 419–72
13. Roberts AB, Sporn MB. 1993. *Growth Factors* 8:1–9
14. Alexandrow MG, Moses HL. 1995. *Cancer Res.* 55:1452–57
15. Cate RL, Donahoe PK, MacLaughlin DT. 1990. See Ref. 290, pp. 179–210
16. Josso N, Cate RL, Picard JY, Vigier B, di Clemente N, et al. 1993. *Recent Prog. Horm. Res.* 48:1–49
17. Massagué J. 1996. *Nature* 382:29–30
18. Mathews LS, Vale WW. 1991. *Cell* 65: 973–82
19. Cheifetz S, Weatherbee JA, Tsang ML-S, Anderson JK, Mole JE, et al. 1987. *Cell* 48:409–15
20. Laiho M, Weis FMB, Massagué J. 1990. *J. Biol. Chem.* 265:18518–24
21. Georgi LL, Albert PS, Riddle DL. 1990. *Cell* 61:635–45
22. Lin L-FH, Doherty DH, Lile JD, Bektesh S, Collins F. 1993. *Science* 260:1130–32
23. di Clemente N, Wilson C, Faure E, Boussin L, Carmillo P, et al. 1994. *Mol. Endocrinol.* 8:1006–20
24. Franzén P, ten Dijke P, Ichijo H, Yamashita H, Schulz P, et al. 1993. *Cell* 75:681–92
25. Yamashita H, ten Dijke P, Franzén P, Miyazono K, Heldin CH. 1994. *J. Biol. Chem.* 269:20172–78
26. ten Dijke P, Ichijo H, Franzén P, Schulz P, Saras J, et al. 1993. *Oncogene* 8:2879–87
27. Cárcamo J, Weis FMB, Ventura F, Wieser R, Wrana JL, et al. 1994. *Mol. Cell Biol.* 14:3810–21
28. Yamashita H, ten Dijke P, Huylebroeck D, Sampath TK, Andries M, et al. 1995. *J. Cell Biol.* 130:217–26
29. Koenig BB, Cook JS, Wolsing DH, Ting J, Tiesman JP, et al. 1994. *Mol. Cell Biol.* 14:5961–74
30. Tsuchida K, Sawchenko PE, Nishikawa S, Vale WW. 1996. *Mol. Cell. Neurosci.* 7:467–78
31. Rydén M, Imamura T, Jörnvall H, Belluardo N, Neveu I, et al. 1996. *J. Biol. Chem.* 271:30603–9
32. Mahony D, Gurdon JB. 1995. *Proc. Natl. Acad. Sci. USA* 92:6474–78
33. Attisano L, Cárcamo J, Ventura F, Weis FMB, Massagué J, et al. 1993. *Cell* 75: 671–80
34. ten Dijke P, Yamashita H, Ichijo H, Franzén P, Laiho M, et al. 1994. *Science* 264:101–4
35. ten Dijke P, Yamashita H, Sampath TK, Reddi AH, Estevez M, et al. 1994. *J. Biol. Chem.* 269:16985–88

36. Liu F, Ventura F, Doody J, Massagué J. 1995. *Mol. Cell. Biol.* 15:3479–86
37. Ebner R, Chen R-H, Lawler S, Zioncheck T, Derynck R. 1993. *Science* 262: 900–2
38. He WW, Gustafson ML, Hirobe S, Donahoe PK. 1993. *Dev. Dyn.* 196:133–42
39. Armes NA, Smith JC. 1997. *Development* 124:3797–804
40. Lin HY, Wang X-F, Ng-Eaton E, Weinberg RA, Lodish HF. 1992. *Cell* 68:775–85
41. Rosenzweig BL, Imamura T, Okadome T, Cox GN, Yamashita H, et al. 1995. *Proc. Natl. Acad. Sci. USA* 92:7632–36
42. Nohno T, Ishikawa T, Saito T, Hosokawa K, Noji S, et al. 1995. *J. Biol. Chem.* 270:22522–26
43. Baarens WM, van Helmaond MJL, Post M, van der Schoot PJCM, Hoogerbrugge JW, et al. 1994. *Development* 120:189–97
44. Attisano L, Wrana JL, Cheifetz S, Massagué J. 1992. *Cell* 68:97–108
45. Mathews LS, Vale WW, Kintner CR. 1992. *Science* 255:1702–5
46. Hoodless PA, Haerry T, Abdollah S, Stapleton M, O'Connor MB, et al. 1996. *Cell* 85:489–500
47. Nishitoh H, Ichijo H, Kimura M, Matsumoto T, Makishima F, et al. 1996. *J. Biol. Chem.* 271:21345–52
48. Nellen D, Affolter M, Basler K. 1994. *Cell* 78:225–37
49. Penton A, Chen YJ, Staehling-Hampton K, Wrana JL, Attisano L, et al. 1994. *Cell* 78:239–50
50. Brummel TJ, Twombly V, Marques G, Wrana JL, Newfeld SJ, et al. 1994. *Cell* 78:251–61
51. Xie T, Finelli AL, Padgett RW. 1994. *Science* 263:1756–59
52. Letsou A, Arora K, Wrana JL, Simin K, Twombly V, et al. 1995. *Cell* 80:899–908
53. Ruberte E, Marty T, Nellen D, Affolter M, Basler K. 1995. *Cell* 80:889–97
54. Wrana JL, Tran H, Attisano L, Arora K, Childs SR, et al. 1994. *Mol. Cell. Biol.* 14:944–50
55. Estevez M, Attisano L, Wrana JL, Albert PS, Massagué J, et al. 1993. *Nature* 365:644–49
56. Ren PF, Lim C-S, Johnsen R, Albert PS, Pilgrim D, et al. 1996. *Science* 274: 1389–91
57. Ebner R, Chen R-H, Shum L, Lawler S, Zioncheck TF, et al. 1993. *Science* 260:1344–48
58. Cheifetz S, Andres JL, Massagué J. 1988. *J. Biol. Chem.* 263:16984–91
59. Wells RG, Yankelev H, Lin HY, Lodish HF. 1997. *J. Biol. Chem.* 272:11444–51
60. Luo KX, Lodish HF. 1997. *EMBO J.* 16:1970–81
61. Souchelnytskyi S, ten Dijke P, Miyazono K, Heldin CH. 1996. *EMBO J.* 15:6231–40
62. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J. 1994. *Nature* 370:341–47
63. Wieser R, Wrana JL, Massagué J. 1995. *EMBO J.* 14:2199–208
64. Attisano L, Wrana JL, Montalvo E, Massagué J. 1996. *Mol. Cell. Biol.* 16:1066–73
65. Charng M-J, Kinnunen P, Hawker J, Brand T, Schneider MD. 1996. *J. Biol. Chem.* 271:22941–44
66. Chen YG, Liu F, Massagué J. 1997. *EMBO J.* 16:3866–76
67. Wiersdorff V, Lecuit T, Cohen SM, Mlodzik M. 1996. *Development* 122: 2153–62
68. Kretzschmar M, Liu F, Hata A, Doody J, Massagué J. 1997. *Genes Dev.* 11:984–95
69. Zou HY, Wieser R, Massagué J, Niswander L. 1997. *Genes Dev.* 11:2191–203
70. Macias-Silva M, Abdollah S, Hoodless PA, Pirone R, Attisano L, et al. 1996. *Cell* 87:1215–24
71. Mathews LS, Vale WW. 1993. *J. Biol. Chem.* 268:19013–18
72. Bassing CH, Yingling JM, Howe DJ, Wang TW, He WW, et al. 1994. *Science* 263:87–89
73. Lawler S, Fen XH, Chen R-W, Maruoka EM, Turck CW, et al. 1997. *J. Biol. Chem.* 272:14850–58
74. Wrana JL, Attisano L, Carcamo J, Zentella A, Doody J, et al. 1992. *Cell* 71: 1003–14
75. Weis-Garcia F, Massagué J. 1996. *EMBO J.* 15:276–89
76. Taylor SS, Radzio-Andzelm E. 1994. *Structure* 2:345–55
77. Feng XH, Derynck R. 1997. *EMBO J.* 16:3912–22
78. Kawabata M, Chytil A, Moses HL. 1995. *J. Biol. Chem.* 270:5625–30
79. Wieser R, Attisano L, Wrana JL, Massagué J. 1993. *Mol. Cell Biol.* 13:7239–47
80. Ullrich A, Schlessinger J. 1990. *Cell* 61: 203–12
81. Suzuki A, Shioda N, Maeda T, Tada M, Ueno N. 1994. *FEBS Lett.* 355:19–22
82. Hirai R, Fujita T. 1996. *Exp. Cell Res.* 223:135–41
83. Sun PD, Davies D. 1995. *Annu. Rev. Biophys. Biomol. Struct.* 24:269–91

84. Daopin S, Piez KA, Ogawa Y, Davies DR. 1992. *Science* 257:369–73
85. Schlunegger MP, Grütter M. 1992. *Nature* 358:430–34
86. Griffith DL, Keck PC, Sampath TK, Rueger DC, Carlson WD. 1996. *Proc. Natl. Acad. Sci. USA* 93:878–83
87. Hinck AP, Archer SJ, Quian SW, Roberts AB, Sporn MB, et al. 1996. *Biochemistry* 35:8517–34
88. Qian SW, Burmester JK, Tsang MLS, Weatherbee JA, Hinck AP, et al. 1996. *J. Biol. Chem.* 261:30656–62
89. Ogawa Y, Schmidt DK, Dasch JR, Chang RJ, Glaser CB. 1992. *J. Biol. Chem.* 267:2325–28
90. Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, et al. 1986. *Nature* 321:779–82
91. Aono A, Hazama M, Notoya K, Taketomi S, Yamasaki H, et al. 1995. *Biochem. Biophys. Res. Commun.* 210:670–77
92. Padgett RW, Wozney JM, Gelbart WM. 1993. *Proc. Natl. Acad. Sci. USA* 90: 2905–9
93. Sampath TK, Rashka KE, Doctor JS, Tucker RF, Hoffman FM. 1993. *Proc. Natl. Acad. Sci. USA* 90:6004–8
94. Boyd FT, Massagué J. 1989. *J. Biol. Chem.* 264:2272–78
95. Laiho M, Weis FMB, Boyd FT, Ignotz RA, Massagué J. 1991. *J. Biol. Chem.* 266:9108–12
96. Ventura F, Doody J, Liu F, Wrana JL, Massagué J. 1994. *EMBO J.* 13:5581–89
97. Tsang ML, Zhou L, Zheng BL, Wenker J, Fransen G, et al. 1995. *Cytokine* 7: 389–97
98. Lin HY, Moustakas A, Knaus P, Wells RG, Henis YI, et al. 1995. *J. Biol. Chem.* 270:2747–54
99. Natsume T, Tomita S, Iemura S, Kinto N, Yamaguchi A, et al. 1997. *J. Biol. Chem.* 272:11535–40
100. Childs SR, Wrana JL, Arora K, Attisano L, O'Connor MB, et al. 1993. *Proc. Natl. Acad. Sci. USA* 90:9475–79
101. López-Casillas F, Cheifetz S, Doody J, Andres JL, Lane WS, et al. 1991. *Cell* 67:785–95
102. Wang X-F, Lin HY, Ng-Eaton E, Downward J, Lodish HF, et al. 1991. *Cell* 67:797–805
103. Gougos A, Letarte M. 1990. *J. Biol. Chem.* 265:8361–64
104. Segarini PR, Seyedin SM. 1988. *J. Biol. Chem.* 263:8366–70
105. López-Casillas F, Payne HM, Andres JL, Massagué J. 1994. *J. Cell Biol.* 124:557–68
106. Zhang L, Esko JD. 1994. *J. Biol. Chem.* 269:19295–99
107. Henis YI, Moustakas A, Lin HY, Lodish HF. 1994. *J. Cell Biol.* 126:139–54
108. Cheifetz S, Massagué J. 1989. *J. Biol. Chem.* 264:12025–28
109. Andres J, DeFalcis D, Noda M, Massagué J. 1992. *J. Biol. Chem.* 267:5927–30
110. Cheifetz S, Bellón T, Calés C, Vera S, Bernabeu C, et al. 1992. *J. Biol. Chem.* 267:19027–30
111. Pepin MC, Beauchemin M, Plamondon J, O'Connor-McCourt MD. 1994. *Proc. Natl. Acad. Sci. USA* 91:6997–7001
112. Kaname S, Ruoslahti E. 1996. *Biochem. J.* 315:815–20
113. Bork P, Sander C. 1992. *FEBS Lett.* 300:237–40
114. Andres JL, Stanley K, Cheifetz S, Massagué J. 1989. *J. Biol. Chem.* 109:3137–45
115. Segarini PR, Rosen DM, Seyedin SM. 1989. *Mol. Endocrinol.* 3:261–72
116. Cheifetz S, Massagué J. 1991. *J. Biol. Chem.* 266:20767–72
117. López-Casillas F, Wrana JL, Massagué J. 1993. *Cell* 73:1435–44
118. Moustakas A, Lin HY, Henis YI, O'Connor-McCourt MD, Lodish HF. 1993. *J. Biol. Chem.* 268:22215–18
119. Ohta M, Greenberger JS, Anklesaria P, Bassols A, Massagué J. 1987. *Nature* 329:539–41
120. Sankar S, Mahooti-Brooks N, Centrella M, McCarthy TL, Madri JA. 1995. *J. Biol. Chem.* 270:13567–72
121. Lastres P, Letamendia A, Zhang HW, Rius C, Almendro N, et al. 1996. *J. Cell Biol.* 133:1109–21
122. Bellón T, Corbí A, Lastres P, Calés C, Cebrián M, et al. 1993. *Eur. J. Immunol.* 23:2340–45
123. Yamashita H, Ichijo H, Grimsby S, Moren A, ten Dijke P, et al. 1994. *J. Biol. Chem.* 269:1995–2001
124. McAllister KA, Grogg KM, Johnson DW, Gallione CJ, Baldwin MA, et al. 1994. *Nat. Genet.* 8:345–51
125. McAllister KA, Baldwin MA, Thukkani AK, Gallione CJ, Berg JN, et al. 1995. *Hum. Mol. Genet.* 4:1983–85
126. Johnson DW, Berg JN, Baldwin MA, Gallione CJ, Marondel I, et al. 1996. *Nat. Genet.* 13:189–95
127. Derynck R, Jarrett JA, Chen EY, Eaton DH, Bell J, et al. 1985. *Nature* 316:701–5
128. Gentry LE, Liobin MN, Purchio AF, Marquardt H. 1988. *Mol. Cell Biol.* 8:4162–68

129. Gentry LE, Webb NR, Lim GJ, Brunner AM, Ranchalis JE, et al. 1987. *Mol. Cell. Biol.* 7:3418–27
130. Böttinger EP, Factor VM, Tsang MLS, Weatherbee JA, Kopp JB, et al. 1996. *Proc. Natl. Acad. Sci. USA* 93:5877–82
131. Miyazono K, Ichijo H, Heldin CH. 1993. *Growth Factors* 8:11–22
132. Olofsson A, Ichijo H, Morén A, ten Dijke P, Miyazono K, et al. 1995. *J. Biol. Chem.* 270:31294–97
133. Morén A, Olofsson A, Stenman G, Sahlin P, Kanzaki T, et al. 1994. *J. Biol. Chem.* 269:32469–78
134. Nunes I, Gleizes PE, Metz CN, Rifkin DB. 1997. *J. Cell Biol.* 136:1151–63
135. Taipale J, Miyazono K, Heldin CH, Keski-Oja J. 1994. *J. Cell Biol.* 124:171–81
136. Schultz-Cherry S, Murphy-Ullrich JE. 1993. *J. Cell Biol.* 122:923–32
137. Forage RG, Ring JM, Brown RW, McInerney BV, Cobon GS, et al. 1986. *Proc. Natl. Acad. Sci. USA* 83:3091–95
138. Lebrun JJ, Vale WW. 1997. *Mol. Cell. Biol.* 17:1682–91
139. Xu JM, McKeehan K, Matsuzaki K, McKeehan WL. 1995. *J. Biol. Chem.* 270:6308–13
140. Ueno N, Ling N, Ying SY, Esch F, Shimasaki S, et al. 1987. *Proc. Natl. Acad. Sci. USA* 84:8282–86
141. Nakamura T, Takio K, Eto Y, Shibai H, Titani K, et al. 1990. *Science* 247:836–38
142. de Winter JP, ten Dijke P, de Vries CJ, van Achterberg TA, Sugino H, et al. 1996. *Mol. Cell. Endocrinol.* 116:105–14
143. Xiao S, Findlay JK. 1991. *Mol. Cell. Endocrinol.* 79:99–107
144. Darland DC, Link BA, Nishi R. 1995. *Neuron* 15:857–66
145. Hemmati-Brivanlou A, Kelly OG, Melton DA. 1994. *Cell* 77:283–95
146. Shimasaki S, Koga M, Esch F, Mercado M, Cooksey K, et al. 1988. *Biochem. Biophys. Res. Commun.* 152:717–23
147. Shimasaki S, Koga M, Esch F, Cooksey K, Mercado M, et al. 1988. *Proc. Natl. Acad. Sci. USA* 85:4218–22
148. Albano RM, Arkell R, Beddington RS, Smith JC. 1994. *Development* 120:803–13
149. Feijen A, Goumans MJ, van den Eijnden-van Raaij AJ. 1994. *Development* 120:3621–37
150. DePaolo LV, Mercado M, Guo YL, Ling N. 1993. *Endocrinology* 132:2221–28
151. Zimmerman LB, De Jesus-Escobar JM, Harland RM. 1996. *Cell* 86:599–606
152. Piccolo S, Sasai Y, Lu B, De Robertis EM. 1996. *Cell* 86:589–98
153. Smith WC, Harland RM. 1992. *Cell* 70:829–40
154. Valenzuela DM, Economides AN, Rojas E, Lamb TM, Nunez L, et al. 1995. *J. Neurosci.* 15:6077–84
155. Sasai Y, Lu B, Steinbeisser H, Geissert D, Gont LK, et al. 1994. *Cell* 79:779–90
156. François V, Solloway M, O'Neill JW, Emery J, Bier E. 1994. *Genes Dev.* 8:2602–16
157. Holley SA, Jackson PD, Sasai Y, Lu B, De Robertis EM, et al. 1995. *Nature* 376:249–53
158. Biehs B, Francois V, Bier E. 1996. *Genes Dev.* 10:2922–34
159. Holley SA, Neul JL, Attisano L, Wrana JL, Sasai Y, et al. 1996. *Cell* 86:607–17
160. Chen R-H, Moses HL, Maruoka EM, Derynck R, Kawabata M. 1995. *J. Biol. Chem.* 270:12235–41
161. Chen R-H, Derynck R. 1994. *J. Biol. Chem.* 269:22868–74
162. Chen F, Weinberg RA. 1995. *Proc. Natl. Acad. Sci. USA* 92:1565–69
163. Wang TW, Donahoe PK, Zervos AS. 1994. *Science* 265:674–76
164. Kawabata M, Imamura T, Miyazono K, Engel ME, Moses HL. 1995. *J. Biol. Chem.* 270:29628–31
165. Okadome T, Oeda E, Saitoh M, Ichijo H, Moses HL, et al. 1996. *J. Biol. Chem.* 271:21687–90
166. Wang TW, Li B-Y, Danielson PD, Shah PC, Rockwell S, et al. 1996. *Cell* 86:435–44
167. Schreiber SL. 1991. *Science* 251:283–87
168. Brillantes A-MB, Ondrias K, Scott A, Kobrinsky E, Ondriasová E, et al. 1994. *Cell* 77:513–23
169. Cameron AM, Steiner JP, Sabatini DM, Kaplin AI, Walensky LD, et al. 1995. *Proc. Natl. Acad. Sci. USA* 92:1784–88
170. Schreiber SL. 1992. *Cell* 70:365–68
171. Sabatini DM, Erdjument-Bromage H, Lui M, Tempst P, Snyder SH. 1994. *Cell* 78:35–43
172. Brown EJ, Albers MW, Shin TB, Ichikawa K, Keith CT, et al. 1994. *Nature* 369:756–58
173. Chen R-H, Miettinen PJ, Maruoka EM, Choy L, Derynck R. 1995. *Nature* 377:548–52
174. Wang TW, Danielson PD, Li BY, Shah PC, Kim SD, et al. 1996. *Science* 271:1120–22

175. Ventura F, Liu F, Doody J, Massagué J. 1996. *J. Biol. Chem.* 271:13931–34
176. Bassing CH, Howe DJ, Segarini PR, Donahoe PK, Wang X-F. 1994. *J. Biol. Chem.* 269:14861–64
177. Okadome T, Yamashita H, Franzén P, Morén A, Heldin C-H, et al. 1994. *J. Biol. Chem.* 269:30753–56
178. Vivien D, Attisano L, Ventura F, Wrana JL, Massagué J. 1995. *J. Biol. Chem.* 270:7134–41
179. Luo KX, Lodish HF. 1996. *EMBO J.* 15:4485–96
180. Willis SA, Zimmerman CM, Li LI, Mathews LS. 1996. *Mol. Endocrinol.* 10:367–79
181. Cárcamo J, Zentella A, Massagué J. 1995. *Mol. Cell. Biol.* 15:1573–81
182. Persson U, Souchelnytskyi S, Franzén P, Miyazono K, ten Dijke P, et al. 1997. *J. Biol. Chem.* 272:21187–94
183. Zhang Y, Feng X-H, Wu R-Y, Derynck R. 1996. *Nature* 383:168–72
184. Brand T, Schneider MD. 1995. *J. Biol. Chem.* 270:8274–84
185. Chen R-H, Ebner R, Derynck R. 1993. *Science* 260:1335–38
186. Sekelsky JJ, Newfeld SJ, Raftery LA, Chartoff EH, Gelbart WM. 1995. *Genetics* 139:1347–58
187. Raftery LA, Twombly V, Wharton K, Gelbart WM. 1995. *Genetics* 139:241–54
188. Savage C, Das P, Finelli AL, Townsend SR, Sun C-Y, et al. 1996. *Proc. Natl. Acad. Sci. USA* 93:790–94
189. Hahn SA, Schutte M, Hoque ATMS, Moskaluk CA, da Costa LT, et al. 1996. *Science* 271:350–53
190. Graff JM, Bansal A, Melton DA. 1996. *Cell* 85:479–87
191. Liu F, Hata A, Baker JC, Doody J, Cárcamo J, et al. 1996. *Nature* 381:620–23
192. Yingling JM, Das P, Savage C, Zhang M, Padgett RW, Wang X-F. 1996. *Proc. Natl. Acad. Sci. USA* 93:8940–44
193. Eppert K, Scherer SW, Ozcelik H, Pirone R, Hoodless P, et al. 1996. *Cell* 86:543–52
194. Lechleider RJ, de Caestecker MP, Dehejia A, Polymeropoulos MH, Roberts AB. 1996. *J. Biol. Chem.* 271:17617–20
195. Chen Y, Lebrun JJ, Vale W. 1996. *Proc. Natl. Acad. Sci. USA* 93:12992–97
196. Imamura T, Takase M, Nishihara A, Oeda E, Hanai J, et al. 1997. *Nature* 389:622–26
197. Nakao A, Afrakhte M, Morén A, Nakayama T, Christian JL, et al. 1997. *Nature* 389:631–35
198. Watanabe TK, Suzuki M, Omori Y, Hishigaki H, Horie M, et al. 1997. *Genomics* 42:446–51
199. Baker JC, Harland RM. 1996. *Genes Dev.* 10:1880–89
200. Topper JN, Cai J, Qiu Y, Anderson KR, Xu YY, et al. 1997. *Proc. Natl. Acad. Sci. USA* 94:9314–19
201. Lagna G, Hata A, Hemmati-Brivanlou A, Massagué J. 1996. *Nature* 383:832–36
202. Thomsen G. 1996. *Development* 122:2359–66
203. Suzuki A, Chang CB, Yingling JM, Wang W-F, Hemmati-Brivanlou A. 1997. *Dev. Biol.* 184:402–5
203a. Chen Y, Bhushan A, Vale W. 1997. *Proc. Natl. Acad. Sci. USA* 94:12938–43
204. Nakao A, Imamura T, Souchelnytskyi S, Kawabata M, Ishisaki A, et al. 1997. *EMBO J.* 16:5353–62
205. Newfeld SJ, Chartoff EH, Graff JM, Melton DA, Gelbart WM. 1996. *Development* 122:2099–108
206. Hata A, Lo RS, Wotton D, Lagna G, Massagué J. 1997. *Nature* 388:82–87
206a. Das P, Maduzia LL, Wang H, Finelli AL, Cho SH, et al. 1998. *Development.* In press
206b. Hudson JB, Podos SD, Keith K, Simpson SL, Ferguson EL. 1998. *Development.* In press
206c. Wisotzkey RG, Mehra A, Sutherland DJ, Dobens LL, Liu X, et al. 1998. *Development.* In press
207. Hata A, Lagna G, Massagué J, Hemmati-Brivanlou A. 1998. *Genes Dev.* 12:186–97
208. Hayashi H, Abdollah S, Qiu YB, Cai JX, Xu YY, et al. 1997. *Cell* 89:1165–73
209. Tsuneizumi K, Nakayama T, Kamoshida Y, Kornberg TB, Christian JL, et al. 1997. *Nature* 389:627–31
210. Patterson GI, Koweek A, Wong A, Yanxia L, Ruvkun G. 1997. *Genes Dev.* 11:2679–90
211. Kim J, Johnson K, Chen HJ, Carroll S, Laughon A. 1997. *Nature* 388:304–8
212. Liu F, Pouponnot C, Massagué J. 1997. *Genes Dev.* 11:3157–67
213. Wu R-Y, Zhang Y, Feng X-H, Derynck R. 1997. *Mol. Cell. Biol.* 17:2521–28
214. Shi YG, Hata A, Lo RS, Massagué J, Pavletich NP. 1997. *Nature* 388:87–93
215. Chen X, Weisberg E, Fridmacher V, Watanabe M, Naco G, et al. 1997. *Nature* 389:85–89
216. Kretzschmar M, Doody J, Massagué J. 1997. *Nature* 389:618–22

217. Lo RS, Chen YG, Shi YG, Pavletich N, Massagué J. 1998. *EMBO J.* 17:996–1005
218. Huang H-C, Murtaugh LC, Vize PD, Whitman M. 1995. *EMBO J.* 14:5965–73
219. Chen X, Rubock MJ, Whitman M. 1996. *Nature* 383:691–96
220. Lai E, Clark KL, Burley S, Darnell JE Jr. 1993. *Proc. Natl. Acad. Sci. USA* 90:10421–23
221. Grieder NC, Nellen D, Burke R, Basler K, Affolter M. 1995. *Cell* 81:791–800
222. Arora K, Dai H, Kazuko SG, Jamal J, O'Connor MB, et al. 1995. *Cell* 81:781–90
223. Eresh S, Riese J, Jackson DB, Bohmann D, Bienz M. 1997. *EMBO J.* 16:2014–22
223a. Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT, Wang XF. 1997. *Mol. Cell. Biol.* 17:7019–28
223b. Zawel L, Dai JL, Buckaults P, Zhou P, Kinzler KW, et al. 1998. *Mol. Cell.* 1:611–17
224. Hannon GJ, Beach D. 1994. *Nature* 371:257–61
225. Datto MB, Li Y, Panus JF, Howe DJ, Xiong Y, Wang X-F. 1995. *Proc. Natl. Acad. Sci. USA* 92:5545–49
226. Reynisdóttir I, Polyak K, Iavarone A, Massagué J. 1995. *Genes Dev.* 9:1831–45
227. Reynisdóttir I, Massagué J. 1997. *Genes Dev.* 11:492–503
228. Datto MB, Yu Y, Wang X-F. 1995. *J. Biol. Chem.* 270:28623–28
229. Li JM, Nichols MA, Chandrasekharan S, Xiong Y, Wang X-F. 1995. *J. Biol. Chem.* 270:26750–53
230. Ritzenthaler JD, Goldstein RH, Fine A, Smith BD. 1993. *J. Biol. Chem.* 268: 13625–31
231. Rossi P, Karsenty G, Roberts AB, Roche NS, Sporn MB, et al. 1988. *Cell* 5:405–14
232. Inagaki Y, Truter S, Ramirez F. 1994. *J. Biol. Chem.* 269:14828–34
233. Riccio A, Pedone PV, Lund LR, Olesen T, Olsen HS, et al. 1992. *Mol. Cell. Biol.* 12:1846–55
234. Keeton MR, Curriden SA, van Zonneveld A-J, Loskutoff D. 1991. *J. Biol. Chem.* 266:23048–52
235. Marigo V, Volpin D, Vitale G, Bressan GM. 1994. *Biochem. Biophys. Res. Commun.* 199:1049–56
236. Iozzo RV, Pillarisetti J, Sharma B, Murdoch AD, Danielson KG, et al. 1997. *J. Biol. Chem.* 272:5219–28
237. Iavarone A, Massagué J. 1997. *Nature* 387:417–22
238. Chang E, Goldberg H. 1995. *J. Biol. Chem.* 270:4473–77
239. Chen Y, Takeshita A, Ozaki K, Kitano S, Hanazawa S. 1996. *J. Biol. Chem.* 271: 31602–6
240. Kerr LD, Miller DB, Matrisian LM. 1990. *Cell* 61:267–78
241. Ohno M, Cooke JP, Dzau VJ, Gibbons GH. 1995. *J. Clin. Invest.* 95:1363–69
242. Riggins GJ, Kinzler KW, Vogelstein B, Thiagalingam S. 1997. *Cancer Res.* 57:2578–80
243. Blenis J. 1993. *Proc. Natl. Acad. Sci. USA* 90:5889–92
244. Davis RJ. 1993. *J. Biol. Chem.* 268: 14553–56
245. Hartsough MT, Mulder KM. 1995. *J. Biol. Chem.* 270:7117–24
246. Howe PH, Dobrowolski SF, Reddy KB, Stacey DW. 1993. *J. Biol. Chem.* 268:21448–52
247. Berrou E, Fontenay-Roupie M, Quarck R, McKenzie FR, Levy-Toledano S, et al. 1996. *Biochem. J.* 316:167–73
248. Chatani Y, Tanimura S, Miyoshi N, Hattori A, Sato M, et al. 1995. *J. Biol. Chem.* 270:30686–92
249. Yamaguchi K, Shirakabe K, Shibuya H, Irie K, Oishi I, et al. 1995. *Science* 270:2008–11
250. Shibuya H, Yamaguchi K, Shirakabe K, Tonegawa A, Gotoh Y, et al. 1996. *Science* 272:1179–82
251. Atfi A, Djelloul S, Chastre E, Davis RR, Gespach C. 1997. *J. Biol. Chem.* 272:1429–32
252. Wang W, Zhou G, Hu MCT, Yao Z, Tan TH. 1997. *J. Biol. Chem.* 272:22771–75
253. Fynan TM, Reiss M. 1993. *Crit. Rev. Oncog.* 4:493–540
254. Border WA, Ruoslahti E. 1992. *J. Clin. Invest.* 90:1–7
255. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, et al. 1992. *Nature* 359:693–99
256. Kulkarni AB, Huh C-G, Becker D, Geiser A, Lyght M, et al. 1993. *Proc. Natl. Acad. Sci. USA* 90:770–74
257. Wahl SM. 1992. *J. Clin. Immunol.* 12:61–74
258. Massagué J, Weis-Garcia F. 1996. In *Cancer Surveys Cell Signalling*, ed. T Pawson, P Parker, 27:41–64. London: ICRF
259. Markowitz SD, Roberts AB. 1996. *Cytokine Growth Factor Rev.* 7:93–102
260. Markowitz S, Wang J, Myeroff L, Parsons R, Sun LZ, et al. 1995. *Science* 268:1336–38

261. Parsons R, Myeroff LL, Liu B, Willson JK, Markowitz SD, et al. 1995. *Cancer Res.* 55:5548–50
262. Myeroff LL, Parsons R, Kim S-J, Hedrick L, Cho KR, et al. 1995. *Cancer Res.* 55:5545–47
263. Lu S-L, Zhang W-C, Akiyama Y, Nomizu T, Yuasa Y. 1996. *Cancer Res.* 56:4595–98
264. Vincent F, Hagiwara K, Ke Y, Stoner GD, Demetrick DJ, et al. 1996. *Biochem. Biophys. Res. Commun.* 223:561–64
265. Akiyama Y, Iwanaga R, Saitoh K, Shiba K, Ushio K, et al. 1997. *Gastroenterology* 112:33–39
266. Kaneko H, Horiike S, Taniwaki M, Misawa S. 1996. *Leukemia* 10:1696–99
267. Knaus PI, Lindemann D, DeCoteau JF, Perlman R, Yankelev H, et al. 1996. *Mol. Cell Biol.* 16:3480–89
268. Park KC, Kim SJ, Bang YJ, Park JG, Kim NK, et al. 1994. *Proc. Natl. Acad. Sci. USA* 91:8772–76
269. Garrigue-Antar L, Muñoz-Antonia T, Antonia SJ, Gesmonde J, Vellucci VF, et al. 1995. *Cancer Res.* 55:3982–87
270. Takagi Y, Kohmura H, Futamura M, Kida H, Tanemura H, et al. 1996. *Gastroenterology* 111:1369–72
271. MacGrogan D, Pegram M, Slamon D, Bookstein R. 1997. *Oncogene* 15:1111–14
272. Schutte M, Hruban RH, Hedrick L, Cho KR, Nadasdy GM, et al. 1996. *Cancer Res.* 56:2527–30
273. Kim SK, Fan YH, Papadimitrakopoulou V, Clayman G, Hittelman WN, et al. 1996. *Cancer Res.* 56:2519–21
274. Lei JY, Zou TT, Shi YQ, Zhou XL, Smolinski KN, et al. 1996. *Oncogene* 13:2459–62
274a. Takaku K, Oshima M, Miyoshi H, Matsui M, Seldin MF, Taketo MM, 1998. *Cell* 92:645–56
275. Uchida K, Nagatake M, Osada H, Yatabe Y, Kondo M, et al. 1996. *Cancer Res.* 56:5583–85
276. Riggins GJ, Thiagalingam S, Rozenblum E, Weinstein CL, Kern SE, et al. 1996. *Nat. Genet.* 13:347–49
277. Storm EE, Huynh TV, Copeland NG, Jenkins NA, Kingsley DM, et al. 1994. *Nature* 368:639–43
278. Storm EE, Kingsley DM. 1996. *Development* 122:3969–79
279. Thomas JT, Lin K, Nandedkar M, Camargo M, Cervenka J, et al. 1996. *Nat. Genet.* 12:315–17
280. Marchuk DA. 1997. *Chest* 111:S79–82
281. Teixeira J, He WW, Shah PC, Morikawa N, Lee MM, et al. 1996. *Endocrinology* 137:160–65
282. Guerrier D, Tran D, Vanderwinden JM, Hideux S, Van Outryve L, et al. 1989. *J. Clin. Endocrinol. Metab.* 68:45–52
283. Knebelmann B, Boussin L, Guerrier D, Legeai L, Kahn A, et al. 1991. *Proc. Natl. Acad. Sci. USA* 88:3767–71
284. Carré-Eusèbe D, Imbeaud S, Harbison M, New MI, Josso N, Picard JY. 1992. *Hum. Genet.* 90:389–94
285. Imbeaud S, Carré-Eusèbe D, Rey R, Belville C, Josso N, et al. 1994. *Hum. Mol. Genet.* 3:125–31
286. Imbeaud S, Faure E, Lamarre I, Mattéi M-G, di Clemente N, et al. 1995. *Nat. Genet.* 11:382–88
287. Faure E, Gouedard L, Imbeaud S, Cate R, Picard JY, et al. 1996. *J. Biol. Chem.* 271:30571–75
288. Imbeaud S, Belville C, Messika-Zeitoun L, Rey R, di Clemente N, et al. 1996. *Hum. Mol. Genet.* 5:1269–77
289. Mishina Y, Rey R, Finegold MJ, Matzuk MM, Josso N, et al. 1996. *Genes Dev.* 10:2577–87
290. Sporn MB, Roberts AB, eds. 1990. *Peptide Growth Factors and Their Receptors*, Vol. 95. Berlin: Springer-Verlag

EXHIBIT III

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A *Xenopus* type I activin receptor mediates mesodermal but not neural specification during embryogenesis

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SUMMARY

Activins and other ligands in the TGFβ superfamily signal through a heteromeric complex of receptors. Disruption of signaling by a truncated type II activin receptor, XActRIIB (previously called XAR1), blocks mesoderm induction and promotes neuralization in *Xenopus* embryos. We report the cloning and characterization of a type I activin receptor, XALK4. Like truncated XActRIIB, a truncated mutant (tXALK4) blocks mesoderm formation both in vitro and in vivo; moreover, an active form of the receptor induces mesoderm in a ligand-independent manner. Unlike truncated XActRIIB, however, tXALK4 does not induce neural tissue. This difference is explained by the finding that tXALK4 does not block BMP4-mediated epidermal specification, while truncated XActRIIB inhibits all BMP4 responses in embryonic explants. Thus, the type I and type II activin receptors are involved in overlapping but distinct sets of embryonic signaling events.

Key words: mesoderm induction, neural induction, activin, BMP4, type I activin receptor, *Xenopus*, TGFβ

INTRODUCTION

In *Xenopus*, as in other vertebrates, the earliest events in the establishment of the body axis involve the formation of the three germ layers: ectoderm, mesoderm and endoderm. The subsequent movements of these layers during gastrulation generate new tissue interactions which lead to the final determination of the body axis (Gilbert, 1992). Many growth factors have been identified that mediate one or more of the steps in this process. Notably, the fibroblast growth factor (FGF) family and members of the transforming growth factor β (TGFβ) superfamily including activins, Vg1 and BMPs have been shown to induce a variety of embryonic tissue types (for reviews, see Klein and Melton, 1994; Harland, 1994; Kessler and Melton, 1994). Activins, Vg1 and several BMPs are expressed maternally as RNA or protein. Activin can induce several types of mesoderm as well as endoderm in ectodermal explants (animal caps) in a concentration-dependent manner (Green and Smith, 1990; Green et al., 1992; Symes et al., 1994; Gamer and Wright, 1995; Henry et al., 1996). It is proposed that activin may act as a morphogen in *Xenopus* embryos, forming a gradient to determine different tissue types along the dorsal-ventral axis (Green and Smith, 1991; Gurdon et al., 1994). Vg1 is a vegetally localized maternal RNA (Weeks and Melton, 1987) that has the ability to rescue a complete axis in UV-ventralized embryos (Thomsen and Melton, 1993). While both activin and Vg1 can induce a whole repertory of embryonic tissues in embryonic explants, BMPs have only been found to mediate the induction of ventral types of mesoderm (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). Recent studies have also suggested that BMP4 is involved in epidermal specification and neural inhibition (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995; Hawley et al., 1995; Xu et al., 1995).

While FGF signals through ligand-induced homodimerization of receptors with intrinsic protein tyrosine kinase activity, activin and BMP4 utilize a heteromeric receptor complex containing at least one molecule from each of the two subfamilies of receptor serine/threonine (ser/thr) kinases (reviewed in Mathews, 1994). Type II receptors (ActRII, TβRII, BMPRII) have been identified either by ligand binding or by association with type I receptors (Mathews and Vale, 1991; Lin et al., 1992; Liu et al., 1995). The type I receptors (also called ALKs – activin receptor like kinase) were originally characterized based on sequence similarity to the type II receptors (ten Dijke et al., 1993; Ebner et al., 1993; ten Dijke et al., 1994a). Both classes of receptors have been implicated in signal transduction. For activin (Attisano et al., 1996; Willis et al., 1996) and TGFβ (Wrana et al., 1992; Wrana et al., 1994), a model for receptor activation has been suggested in which type I receptors become phosphorylated, in a type II-dependent manner, at a conserved glycine/serine-rich sequence in the juxtamembrane region; it is likely that BMP receptors become activated in a similar fashion (Liu et al., 1995). Although activin and TGFβ display no apparent binding or functional interactions with each other's receptors, it appears that there may be significant sharing of receptors between activin and BMPs. As evidence that a particular type II receptor can interact with multiple ligands, the activin type II receptor, ActRII, has been reported to bind to, and potentially signal in response to, BMP7 as well as activin (Yamashita et al., 1995);

similarly, the type II BMP receptor, BMPRII, binds both BMP2 and BMP7 (ten Dijke et al., 1994b; Liu et al., 1995). Comparable data indicate that both activins and BMPs may signal through receptor complexes containing a single type I receptor ALK2 (ActRI), together with ActRII (Yamashita et al., 1995). Finally, it has been proposed that individual type I receptors may associate with different type II receptors; ALK2, ALK3 (BMPR-IA) and ALK6 (BMPR-IB) have been found to interact with both ActRII and BMPR-II (ten Dijke et al., 1994a; Liu et al., 1995; Yamashita et al., 1995). To date, neither the relative binding affinities nor the signaling capacities of the various ligand-receptor complexes have been accurately determined.

Several receptor ser/thr kinases have been cloned from *Xenopus*, including type II activin and type I BMP4 receptors (Mathews et al., 1992; Hemmati-Brivanlou et al., 1992; Graff et al., 1994). Expression of truncated forms of these receptors has revealed that deletion of the intracellular domains yields molecules that can inhibit signaling in a dominant negative manner, and the results have also implicated these signaling pathways in mesoderm induction and patterning (Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994; Suzuki et al., 1994). Consistent with the ventralizing activity of BMP4 ligand, truncated type I BMP4 receptor converts ventral mesoderm to dorsal mesoderm, implying that the wild-type receptor is involved in ventral mesoderm specification (Graff et al., 1994; Suzuki et al., 1994). In the case of type II activin receptor, the truncated mutant blocks all mesoderm formation, suggesting a requirement for activin. However, this truncated receptor may block more than one ligand and the role of activin in mesoderm induction remains controversial (Schulte-Merker et al., 1994; Hemmati-Brivanlou and Thomsen, 1995). In addition to mesoderm induction, experiments with these receptors have uncovered previously unsuspected roles for their ligands in early development. Truncated versions of both type II activin receptor and type I BMP4 receptor can divert the ectoderm from an epidermal to a neural fate (Hemmati-Brivanlou and Melton, 1992, 1994; Xu et al., 1995). These studies have shown that members of this growth factor family are not only important in mesoderm formation, but also play an inhibitory role in the formation of the neural tissues. Since studies in cell culture indicate that each type II receptor may interact with several type I receptors to bind different ligands, determining the function of each ligand in frog embryos will require the cloning and functional characterization of the complete family of related ligands and receptors.

In this paper, we report the cloning and functional study of an activin type I receptor, which we have named XALK4 (for *Xenopus* ALK4). Consistent with a role in early embryonic development, this receptor is expressed maternally and is distributed widely in early embryos. The type II activin receptor, previously called XAR1 and renamed here XActRIIB (to remain consistent with the original nomenclature used for these receptors), is also expressed in most of the cells of the early embryo. As was the case with the type II activin receptor (Hemmati-Brivanlou et al., 1992; Mathews et al., 1992), ectopic expression of wild-type XALK4 induces mesoderm in embryonic explants, and truncated XALK4 (tXALK4) blocks mesoderm formation both in explants and in the embryo. Surprisingly, however, in contrast to truncated XActRIIB (previously referred to as ΔIXAR1 and here, because of the new nomenclature, tXActRIIB), tXALK4 inhibits the mesoderm-inducing activity of BMP4 without concurrent neuralization of animal cap explants. In addition, truncated XALK4 does not block epidermal induction by BMP4 in the context of dissociated animal cap cells. Moreover, while wild-type XALK4 rescues mesoderm induction by activin in the presence of tXALK4, it cannot rescue mesoderm induction by BMP4. These results uncover for the first time distinct functions of the two types of activin receptors in the formation of different tissue types in *Xenopus*. They also point to a possible difference between the mechanisms of mesodermal and epidermal induction by BMP4 ligand.

MATERIALS AND METHODS

Cloning and sequencing of XALK4 type I activin receptor and construction of mutants

Two degenerate oligos, CCGGAATTCA(C/T)(A/C)G(G/A/C)-GA(T/C)(A/T/C)TNAA(A/G)(A/T)C and CCGAAGCTTA(C/T)-(C/T)TCNGGNGCCAT(A/G)TA, coding for peptide sequences HRD(L/F/I)KS and YMAPEV conserved within serine/threonine kinase domain, were used for PCR cloning of type I activin receptor. A fragment of 180 bp sequence bearing the highest homology to kinase domain of type I activin receptor was used to screen 0.6×10^6 plaques of a *Xenopus* oocyte library (gift of Dr P. Klein, University of Pennsylvania). Four independent clones were isolated, one encoding full-length type I activin receptor XALK4. The sequence was obtained with dideoxynucleotide sequencing method (Sanger et al., 1977) and sequence alignment of ALK4 with other receptors was made with DNA Star, MegAlign, program. Truncated XALK4 was constructed by PCR using the following two primers: GGAGATC-TACCATGGCGGAGCTACCGGCC and GGAGATCTTCA-CATTTCACATGATGGATCC. The PCR fragment encoded N-terminal 164 amino acids of XALK4, including the extracellular and transmembrane domains of the receptor. The *Bgl*II-digested fragment was inserted into the *Bgl*II site of pSP64T vector (Krieg and Melton, 1984). ALK4 and ALK-T206E were constructed as described elsewhere (Willis et al., 1996) and cloned into the *Bgl*II site of pSP64T.

Embryos, RNA preparation, microinjection and animal cap explants

Xenopus embryos, both pigmented and albino, were obtained as previously described (Hemmati-Brivanlou and Harland, 1989). Embryonic stages were determined as described in Nieuwkoop and Faber (1967). The dorsal side of embryos was determined according to animal pole pigmentation and blastomere size at the 4- to 8-cell stage. (Dorsal cells are lighter and smaller than ventral cells.) RNAs encoding wild-type and mutant receptors were synthesized with linearized templates derived from pSP64T vector, using SP6 polymerase (Ambion mMessage mMachine kit). For tXActRIIB and tBR, the linearization of templates was done as described previously (Hemmati-Brivanlou and Melton, 1992; Graff et al., 1994). For tXALK4, as well as for ALK4 and ALK4-T206E, the templates were linearized with *Xba*I. BMP4 RNA was obtained as previously described (Hemmati-Brivanlou and Thomsen, 1995). The RNAs were then injected into the animal poles or marginal zones of early stage embryos. Amount of injected in vitro synthesized RNAs and sites of injection are as described in the Results section. Animal cap explants were removed with hair knives at late blastula stages and allowed to grow until control sibling embryos reached either gastrula or neurula stages. Total RNA was then extracted and analyzed with RT-PCR. In experiments with activin induction, activin RNA was injected at 30 ng into mature oocytes and the oocyte-conditioned medium was collected 3

days later (Kessler and Melton, 1995). A 1:500 dilution of this conditioned medium was used.

RT-PCR assay

RT-PCR assay was performed as previously described (Wilson and Melton, 1994), with the modification that random hexamers rather than oligo(dT) were used to prime reverse transcription. Primers for EF1-α, muscle actin, Xbra and NCAM were described in Hemmati-Brivanlou and Melton (1994). Primers for Xhox-3, globin (Hemmati-Brivanlou and Thomsen, 1995), epidermal keratin (Wilson and Hemmati-Brivanlou, 1995) and NRP-1 (Lamb and Harland, 1995) were as previously described. For XALK4, the following primers were used: XALK4-U: 5′-GCGGAGCTACCGGCCTTCTTC-3′ and XALK4-D: 5′-TGGGATTGCAATAACAGCTAC-3′ and the PCR conditions were: 94°C, 30 seconds; 55°C, 1 minute; 72°C, 30 seconds; for 25 cycles.

Whole-mount in situ hybridization and immunochemistry

Whole-mount in situ hybridization was performed as described (Hemmati-Brivanlou et al., 1990; Harland, 1991). Antisense digoxigenin-labeled Xbra was obtained as previously described (Hemmati-Brivanlou and Melton, 1992). XALK4 antisense probe was synthesized with T7 RNA polymerase with *Eco*RI linearized pBluescript template (Stratagene) containing entire XALK4 coding sequence. Whole-mount antibody staining was performed as described by Hemmati-Brivanlou and Harland (1989). Three antibodies were used: Tor70.1 for notochord staining (Bolce et al., 1992), 12/101 for muscle (Kintner and Brockes, 1984) and 6F11, which is a neural antigen-specific antibody (A gift from Dr W. Harris, UCSD). Tor70.1 and 12/101 were monoclonal antibodies used at 1:500 dilution. 6F11 was from the hybridoma-conditioned medium and was used at 1:1 dilution. The secondary antibody was a goat anti-mouse IgG coupled to horseradish peroxidase (Jackson Laboratories) and was used at 1:250 dilution.

Cell dissociation and reaggregation

The animal pole cells were dissociated in Ca^{2+}/Mg^{2+}-free medium at stage 9-10 as described before (Grunz and Tacke, 1989; Wilson and Melton, 1994). Dissociated cells were reaggregated immediately or after 4 hours and then incubated until control sibling embryos reached late neurula stages. Purified recombinant human BMP4 protein (Gift of Genetic Institute) was used on dissociated cells at 50 ng/ml.

RESULTS

Isolation of a *Xenopus* XALK4 cDNA

To isolate *Xenopus* TGF-β receptors, we performed a PCR amplification of first strand cDNA made from oocyte RNA. The primers were designed to hybridize to the conserved region of all receptor serine/threonine kinases. A fragment of 180 bp whose sequence showed the highest homology to the mammalian type I activin receptor, ALK4 (activin receptor-like kinase, also called ActRIB), was used to screen a maternal oocyte library. A screen of 0.6×10^6 plaques yielded eleven positives containing four different clones, of which one contained a 3 kb insert encoding the full-length receptor. The predicted protein sequence of this clone is shown in Fig. 1A. There are seven conserved cysteine residues in the extracellular domain at intervals characteristic of all type I receptors and a single ser/thr kinase domain following the putative transmembrane region. The sequence shows greater than 85% identity to all ALK4 mammalian, with highest conservation in the cytoplasmic kinase domain (Fig. 1A). Notably, the glycine/serine-rich domain (GS domain) is conserved between the frog gene and all other type I receptor genes; this region has been proposed to play an important role in signal transduction by type I activin receptors. Based on this homology, we named the frog gene *XALK4*. RT-PCR and in situ analysis indicates that *XALK4* is present maternally and expressed widely throughout early embryogenesis, such as in mesodermal and ectodermal tissues (Fig. 1B and data not shown). Thus both the type I and type II activin receptors are expressed from the first cell cycle onward.

To perform functional studies with ALK4, we have utilized mutant forms of both the human *ALK4* and frog *XALK4* genes in early *Xenopus* embryogenesis. Fig. 1C shows a schematic drawing of the receptors used in this study. In addition to wild-type *Xenopus* and human genes, we examined a mutant generated by truncation of most of the intracellular domain (tXALK4), and a point mutant in the GS domain (ALK4-T206E), which can induce expression of activin-dependent genes in the absence of ligand in cell culture (Willis et al., 1996). Because tXALK4 retains intact extracellular ligand-binding sequences, but is impaired in its capacity to transduce signals, it can presumably interfere with the wild-type receptors in a dominant fashion by competing for ligands and type II receptors. Because ALK4-T206E signals without ligand, it represents a constitutively active receptor.

Effects on mesoderm induction of wild-type and mutant ALK4

Because ALK4-T206E has been characterized by gain-of-function studies (Willis et al., 1996) in mammalian cells, we began the functional characterization of ALK4 by testing the effects of this mutant in early embryos. Although this set of experiments was performed using human wild-type and mutant ALK4, the wild-type *Xenopus* clone had identical function in all assays (not shown). We injected 2 ng of in vitro synthesized ALK4 or ALK4-T206E RNA into animal poles of 2-cell embryos. Animal caps were explanted at mid-blastula, incubated in the presence or absence of activin and assayed at late neurula stages for both morphology and tissue-specific molecular markers (Fig. 2). In the presence of activin uninjected explants, as well as explants expressing ALK4 and ALK4-T206E, elongated and expressed the mesodermal markers Xbra and muscle actin. In the absence of activin, ALK4-injected caps showed weak signs of elongation and induction of only the general mesoderm marker Xbra. This weak induction without ligand presumably reflects a low level of basal kinase activity; a similar effect has been observed with other serine/threonine kinase receptors (e.g. Mathews et al., 1992; Hemmati-Brivanlou et al., 1992). Caps expressing ALK4-T206E elongated strongly and expressed both Xbra and muscle actin, suggesting that it acted as a constitutively active receptor that could signal in a ligand-independent manner.

Inhibition of mesoderm induction by activin and BMP4 using truncated XALK4

In *Xenopus*, at least two other receptor ser/thr kinases have been cloned: the type II activin receptor XActRIIB and a type I BMP4 receptor (BMPRI, Hemmati-Brivanlou et al., 1992; Graff et al., 1994). Because receptors in this class act as heteromers, truncation mutants can act as dominant inhibitors of signaling, and this strategy has been successfully used to study

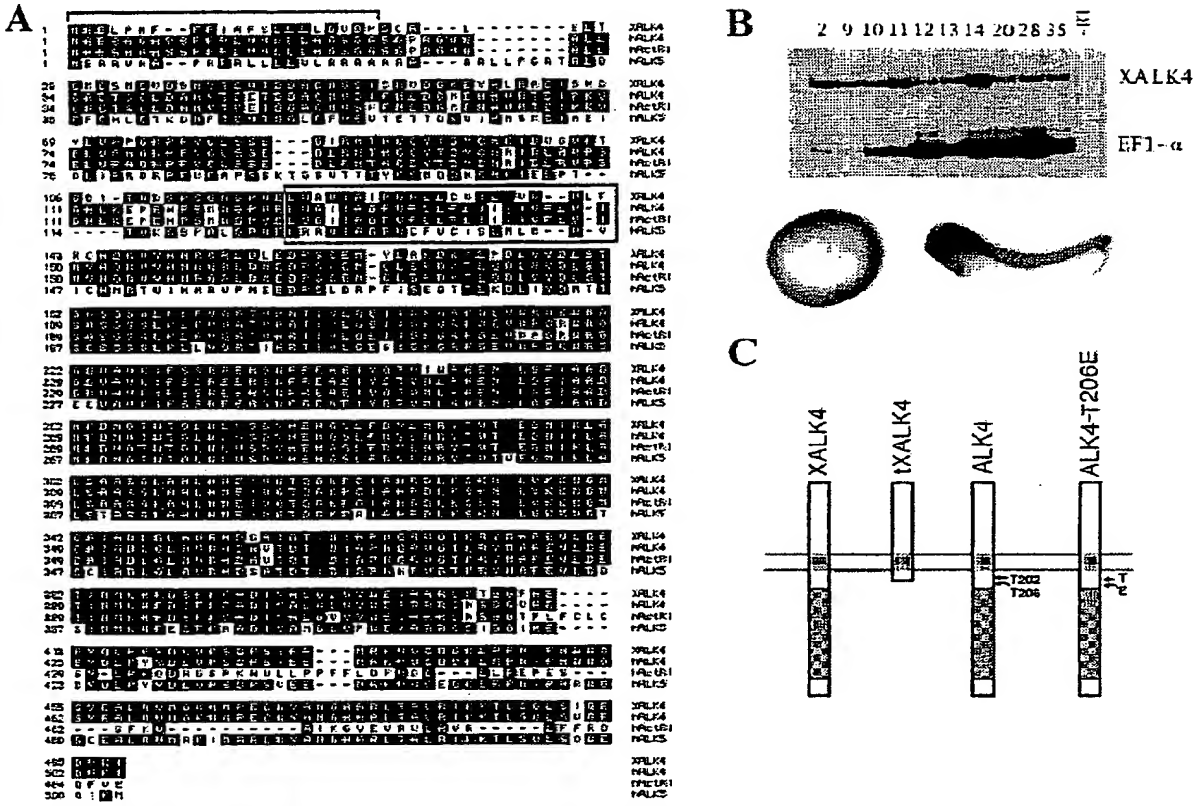


Fig. 1. Wild-type and mutant type I activin receptors. (A) Protein sequence alignment between *Xenopus* ALK4 (XALK4) and three human type I receptors: ALK4, ActRI and ALK5. The signal sequence is overlined and the transmembrane domain is boxed. The cytoplasmic domain starts with amino acid (aa) 143, the GS domain contains the GS core sequence at aa 182-187. (B) XALK4 expression pattern. Top, RT-PCR analysis of XALK4 at different developmental stages; stage 2, 2-cell stage; stages 9 to 11, gastrula stages; stages 12 to 20, neurula stages; stage 28 tailbud stage; stage 35, tadpole. XALK4 is expressed maternally and persists during development. Bottom: in situ hybridization of XALK4 at gastrulation (left, vegetal view with dorsal lip at the top) and tailbud (right, anterior at the left) stages, showing uniform expression in dorsal and ventral sides at early stage and expression in many tissues in late embryos. (C) Schematic presentation of the wild-type and mutant receptors used in this study. tXALK4 contains the first 164 aa of XALK4, which includes the extracellular and transmembrane domains, but excludes GS and kinase domains. GS domain of ALK4 contains Thr at positions 202 and 206, which have been shown to be important for its function in cell culture assays. ALK4-T206E mutant induces transcriptional responses in a ligand-independent manner in cell culture (Willis et al., 1996).

the functions of XActRIIB and BMPRI in embryogenesis (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Melton, 1994; Graff et al., 1994; Suzuki et al., 1994; Xu et al., 1995). To compare the functions of these receptors with type I activin receptor, we generated a truncated *XALK4* gene with a deletion of its kinase domain (tXALK4, see Fig. 1B) and studied the role of this receptor in mesoderm induction by activin and the related factor BMP4.

Embryos were injected with 1 ng of in vitro synthesized receptor RNAs into the animal pole of each blastomere at the 2-cell stage. At blastula stages, animal pole explants were removed and cultured with or without inducing factors. Total RNA was harvested at either gastrula or neurula stage, and RT-PCR was used to assay for expression of mesodermal markers (Fig. 3). As previously demonstrated, activin induced dorsal mesoderm in animal cap explants, as revealed by the expression of muscle actin as well as Xbra (compare lane 1 with lane 5 in Fig. 3A). Injection of tXALK4 blocked mesoderm induction by this ligand (compare lanes 5 and 6 in Fig. 3A). This result, in agreement with observations made in cell culture systems (Wrana et al., 1994), shows that activin signal transduction requires an intact type I receptor with an active cytoplasmic kinase domain. The truncated type II receptor, tXActRIIB, also completely blocked activin induction of these markers, while truncated BMPRI (tBR) did not (Fig. 3A and Graff et al., 1994). There was, however, a somewhat lowered level of expression of Xbra in the presence of tBR. This partial effect has been observed previously (Schmidt et al., 1995; Graff et al., 1994) and could be due to dorsalization of induced mesoderm.

Because tXActRIIB could block both activin and BMP4 signaling (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Thomsen, 1995), we asked whether signaling by BMP4 could also be inhibited by truncated XALK4. Previously it has been shown that BMP4 can induce ventral mesoderm in animal cap explants and can ventralize the dorsal marginal zone (Koster et al., 1991; Dale et al., 1992; Jones et al., 1992; Fainsod et al., 1994; Suzuki et al., 1994; Hemmati-

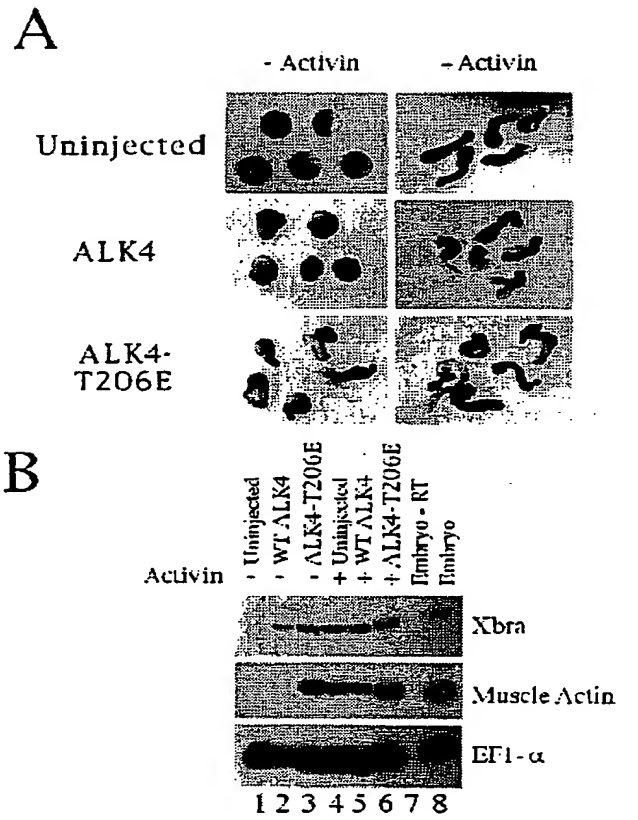


Fig. 2. Mesoderm induction assay by wild-type and mutant ALK4 in ectodermal explants. (A) Morphology of animal caps expressing the different forms of ALK4. Embryos were injected with RNAs encoding wild-type ALK4 or a constitutively active point mutant ALK4-T206E. Caps dissected at blastula stages 8 to 9 were allowed to grow in the absence (left panels) or presence (right panels) of activin until sibling control embryos reached neurula stage 18. (B) Expression of mesoderm-specific markers in animal caps injected with different receptor RNAs. Xbra is an early pan-mesodermal marker, muscle actin is a marker of paraxial mesoderm. Lanes 1-3, caps incubated in buffer alone; lanes 4-6, caps incubated in the presence of activin. Lanes 1 and 4, uninjected control caps; lanes 2 and 5, caps injected with wild-type ALK4 RNA; lanes 3 and 6, caps injected with ALK4-T206E RNA; lanes 7 and 8, whole-embryo controls, in the absence (lane 7) or presence (lane 8) of reverse transcriptase in the RT-PCR reaction. While wild-type ALK4 is a weak mesoderm inducer, ALK4-T206E induces mesoderm in animal caps in the absence of added activin.

Brivanlou and Thomsen, 1995). We injected 0.5 ng in vitro synthesized BMP4 RNA into 2-cell-stage embryos, either alone or together with different receptor RNAs. RT-PCR analysis of animal cap explants expressing BMP4 at gastrula stages revealed that mesodermal markers Xbra, Xwnt8 and Xhox3 were all induced. At later tadpole stages, the ventral mesoderm marker αT1-globin was also expressed (Fig. 3B, lane 5). In contrast, when BMP4 was coinjected with tXALK4 RNA, the expression of all these markers was blocked (compare lane 5 with 6). As a control, we showed that both tBR and tXActRIIB blocked mesoderm induction by BMP4 (Fig. 3B, lanes 7 and 8).

Because ALK4 has not been observed to mediate BMP4-induced transcriptional responses in mammalian cells (L. S. M., unpublished data), we asked whether the block to activin and BMP signaling was specifically due to inhibition of ALK4 activity. We performed rescue experiments by coinjecting wild-type XALK4 or XActRIIB along with tXALK4 (Fig. 4). Coexpression of XALK4 with tXALK4 rescued mesoderm induction by activin, as measured by expression of muscle actin. In contrast, mesoderm induction by injected BMP4 RNA could not be rescued by XALK4. Expression of both early and late markers remained unresponsive to BMP4 in embryos injected with tXALK4, even in the presence of wild-type XALK4. Surprisingly coexpression of wild-type activin type II receptor XActRIIB did reverse the suppressive effect of tXALK4 on mesoderm induction by BMP4, though it did not rescue activin-induced mesoderm formation. The different effect of rescue by wild-type receptors suggests that XALK4 and XActRIIB are differentially involved in mesoderm induction by activin and BMP4. The inhibition of BMP4-mediated mesoderm induction by tXALK4 may result from its titration of a type II activin receptor employed in both pathways.

To see if mutant ALK4 can non-specifically inhibit mesoderm induction by other growth factors, we examined the effect of tXALK4 on FGF-induced mesoderm formation. As described previously, bFGF induced expression of the mesodermal markers Xbra and Xwnt8, but only weak expression of muscle actin and weak elongation (Fig. 3C and data not shown). None of the truncated receptor ser/thr kinases affected bFGF induction of Xbra and muscle actin, demonstrating that inhibition by tXALK4 is specific to members of the TGFβ family such as BMP4 and activin, and that mesoderm induction by bFGF is unaffected.

Involvement of XALK4 in mesoderm formation in vivo

To determine whether XALK4 is involved in mesoderm formation in the context of the embryo, we injected 2 ng of either tXALK4 or ALK4-T206E RNA into the marginal region of one blastomere of 2-cell-stage albino embryos. The embryos were allowed to develop to early gastrula stages before they were fixed and assayed for Xbra expression by whole-mount in situ hybridization (Fig. 5A-F). In the control uninjected early gastrula, Xbra is expressed in a ring around the equator (Smith et al., 1991, Fig. 5A,D). Injection of tXALK4 RNA blocked Xbra expression in half the circumference of the embryo, resulting in a half ring expression pattern (Fig. 5B,E). This result parallels the observations made with tXActRIIB and demonstrates that truncated form of both activin receptors can block endogenous mesoderm formation. In contrast, the constitutively active receptor, ALK4-T206E, enhanced Xbra expression, expanding the pattern of staining into the animal pole in the injected half (Fig. 5C,F). Together, these results implicate XALK4 in mesoderm induction in vivo.

Axial defects in embryos overexpressing truncated XALK4

As XALK4 is involved in mesoderm formation both in animal cap explants and in vivo, we wanted to examine the conse-

quence of tXALK4 on the late phenotype of embryos. We injected 1 ng of the truncated XALK4 RNA into the marginal zone of each blastomere at the 4-cell stage. Over 85% of injected embryos displayed defects in axial structures; 50% had no discernible axis (Fig. 6; Table 1). These embryos had neither heads nor tails, and many developed into 'bubble embryos' (compare injected embryos with controls in Fig. 6). These embryos were very similar to those injected with truncated type II receptor (Hemmati-Brivanlou and Melton, 1992). In that case, the embryos had dramatically reduced mesodermal tissues. We therefore examined the effect of tXALK4 on mesodermal tissues by staining with antibodies against muscle (12/101) and notochord (Tor 70.1). As shown in Fig. 6, expression of both muscle (Fig. 6B) and notochord (Fig. 6C) markers was severely reduced in tXALK4-injected embryos. We also found that neural tissue, detected with the antibody against a neural-specific antigen, was reduced, although to a lesser extent (Fig. 6D). Interestingly, the same phenotype can be obtained if only the two dorsal, but not ventral, blastomeres are injected in the marginal zone or vegetal pole (data not shown). This observation parallels the one made for tXActRIIB (A. H. B, unpublished data) and provides, in agreement with previous observation, further evidence that signals responsible for the establishment of the dorsal axis are derived from the dorsal vegetal blastomeres.

To exclude the possibility that the loss of mesoderm is due to cell death caused by tXALK4 injection, we injected either 100 pg of nuclear-β-Gal alone or nuclear-β-Gal with tXALK4 in a single vegetal blastomere at the 8-cell stage. The comparison of the number of stained nuclei between the control embryos injected with β-Gal alone, versus embryos expressing

Table 1. Axial defect in embryos injected with truncated XALK4 RNA

	No axial structures	Partial axial defects	Normal embryos	Total
No. of embryos	115	87	29	231
Percent	50	37	13	100

Embryos were injected with 1 ng truncated XALK4 RNA in the equatorial region of each blastomere at the 4-cell stage. Embryos were allowed to develop until control siblings reached tailbud stages, when the injected embryos were scored for phenotype and molecular markers. The 'no axial structures' phenotype corresponds to embryos with absent or drastically reduced axial mesodermal marker expression. These embryos were also called 'bubble embryos'. The embryos that showed 'partial axial defects' retained about 20-70% muscle or notochord compared with wild-type uninjected controls and were defective in both head and tail. This division into extreme and partial defects is arbitrary in that a range of defect is observed within each class.

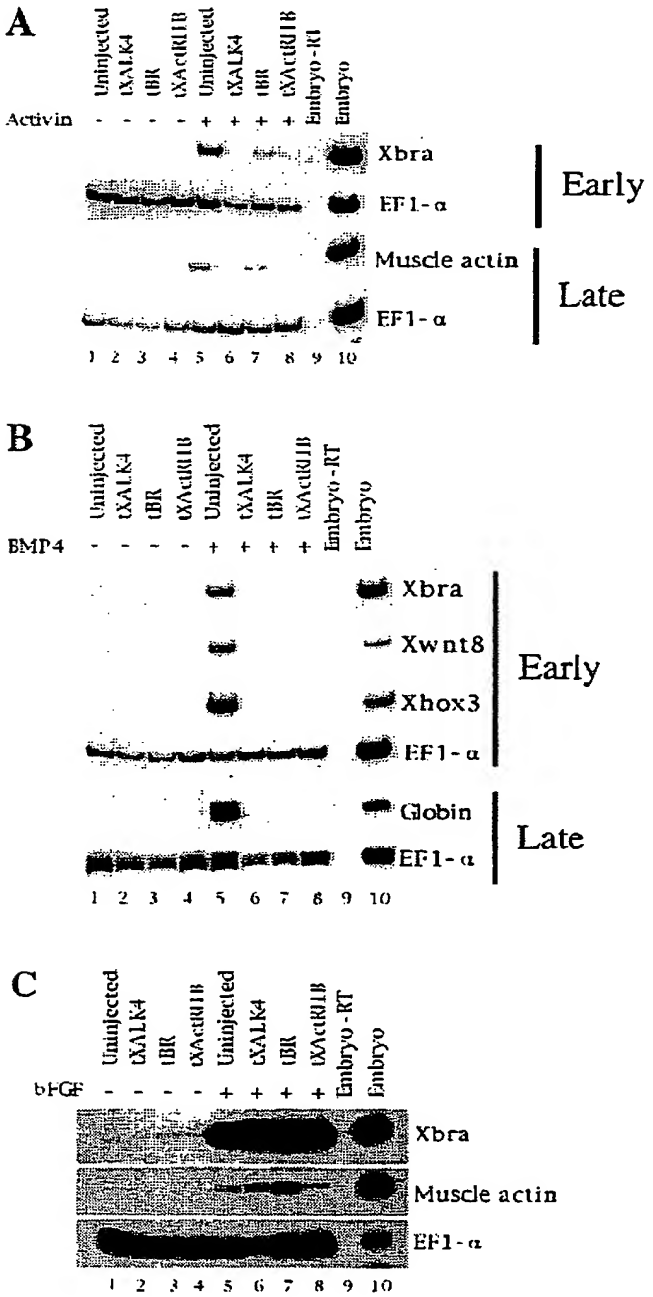


Fig. 3. Truncated XALK4 inhibits mesoderm induction by activin and BMP4. (A) tXALK4 inhibits mesoderm induction by activin. Embryos were injected with tXALK4, tXActRIIB or tBR RNAs at 2-cell stage and animal cap explants were dissected at blastula stages 8 to 9. The caps were either incubated alone (lanes 1 to 4), or with activin (lanes 5 to 8). Total RNA was assayed at either gastrula stage 11 (top panels, 'Early') or tailbud stage 28 (bottom panels, 'Late') by RT-PCR for expression of mesodermal-specific markers. Lanes 1 and 5, uninjected controls; lanes 2 and 6, injected with tXALK4; lanes 3 and 7, injected with tBR; lanes 4 and 8, injected with tXActRIIB. Lane 9 is a negative control without reverse transcriptase in the RT-PCR reaction, and lane 10 is a positive control using RNA extracted from whole embryos at either gastrula (top panel) or tailbud (lower panel) stages. (B) tXALK4 inhibits mesoderm induction by BMP4. BMP4 RNA was injected alone or coinjected with the truncated receptor RNAs. Explants were assayed as described above. Xwnt8 and Xhox3 are early markers of ventroposterior mesoderm. Globin is a marker of ventral mesoderm. (C) tXALK4 does not inhibit mesoderm induction by bFGF. Late blastula explants were incubated in the absence (lanes 1 to 4) or presence (lanes 5 to 8) of 100 ng/ml bFGF protein, and assayed when sibling controls reached late neurula stages as described above.

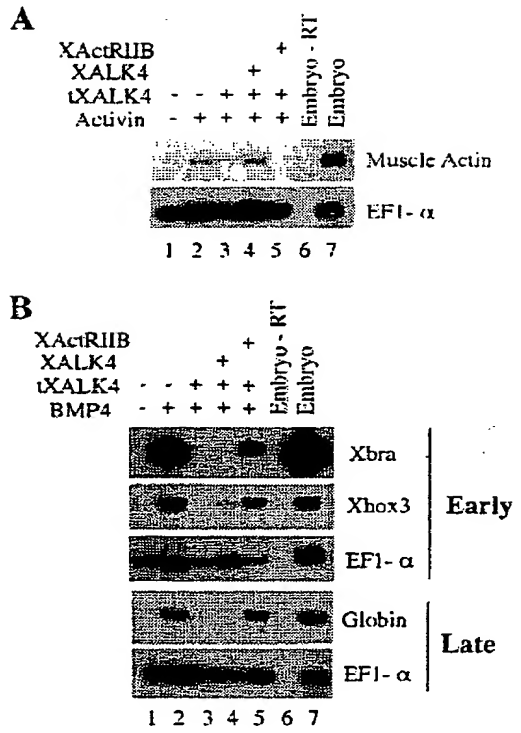


Fig. 4. Wild-type XALK4 receptor rescues tXALK4 blocked activin, but not BMP4, mesoderm induction. Ectodermal explants derived from embryos injected with tXALK4 RNA alone or coinjected with tXALK4 and XALK4 or XActRIIB RNAs were assayed for expression of mesodermal markers. Lane 1, uninjected control explants in buffer alone; lanes 2-5, explants incubated with activin (A) or injected with 0.5 ng BMP4 RNA (B). Lane 3 is animal caps injected with 2 ng tXALK4 RNA, lane 4 is caps coinjected with 2 ng tXALK4 and 0.5 ng XALK4 RNA, and lane 5 is caps coinjected with 2 ng tXALK4 and 0.5 ng XActRIIB RNA. (A) Induction of muscle actin by activin, assayed when control siblings reached tailbud stage 28, was rescued by coinjection of wild-type XALK4 RNA, but not by XActRIIB. (B) Expression of BMP4-induced early mesodermal marker Xbra at control sibling gastrula stage 11 and the late marker globin at stage 28 could not be rescued by co-expression of XALK4, but was rescued by wild-type XActRIIB receptor.

both β-Gal and tXALK4 revealed comparable number of cells and thus suggested that the injected cells did not die (data not shown). To control for the specificity of the tXALK phenotype, we coinjected tXALK4 with wild-type XALK4 RNA in the marginal zone of two dorsal blastomeres of 4-cell embryos. We found that as little as 100 pg of wild-type XALK4 RNA was sufficient to rescue embryos injected with 2 ng tXALK4 RNA and restore the body axis (Fig. 6E), thus demonstrating the specificity of the observed phenotype.

Truncated type I activin receptor does not neuralize ectodermal explants

When signaling was blocked by overexpression of the truncated type II activin receptor in animal cap explants, neural fate was revealed; moreover injection of tXActRIIB into the animal pole resulted in embryos with increased neural structures (cement glands and eyes; Hemmati-Brivanlou and Melton, 1994). These findings suggested that neural fate might be under inhibitory control, and that this inhibitory signaling could be blocked by tXActRIIB. Recent studies indicate that the endogenous neural inhibitor and epidermal inducer is BMP4 or perhaps a related BMP (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995; Hawley et al., 1995; Xu et al., 1995). Because truncated forms of both XALK4 and XActRIIB blocked mesoderm induction by activin and BMP4 (see above), we asked whether tXALK4 could also induce neural tissue in animal cap explants. We injected 2 ng of synthetic RNAs encoding tXALK4, tXActRIIB or tBR, into the animal poles of 2-cell-stage embryos. Expression of neural markers was assayed in animal caps when control sibling embryos reached mid-neurula stage by RT-PCR. Both NCAM (Kintner and Melton, 1987) and NRP-1 (Richter et al., 1990), general neural-specific markers, were induced by tBR or tXActRIIB as previously reported (Xu et al., 1995; Hemmati-Brivanlou and Melton, 1994, Fig. 7A). In contrast, animal caps from embryos injected with the tXALK4 receptor did not express these markers (Fig. 7A), although mesoderm induction by both activin and BMP4 was inhibited at the same dose of RNA (Fig. 3). Increasing the amount of tXALK4 RNA did not neuralize the animal caps (data not shown).

tXALK4 does not block epidermal induction by BMP4

BMP4 is a strong candidate to be the neural inhibitor and epidermal inducer revealed by the neuralizing effects of the truncated type II activin receptor (Wilson and Hemmati-Brivanlou, 1995). When animal caps are dissociated in Ca^{2+}/Mg^{2+}-free medium for 3 to 4 hours before reaggregation, they acquire a neural fate (Grunz and Tacke, 1989); addition of BMP4 to the dissociated cells restores epidermal differentiation (Wilson and Hemmati-Brivanlou, 1995). Expression of tXActRIIB blocks this epidermalizing activity, causing dissociated cells to express neural markers even in the presence of BMP4 (Wilson and Hemmati-Brivanlou, 1995 and Fig. 7B). Since both tXActRIIB and tXALK4 were able to block BMP4-induced mesoderm formation, and yet expression of tXALK4 did not induce neural markers, we asked whether tXALK4 could render ectodermal cells resistant to epidermalization by BMP4. Animal caps were dissociated at blastula stage and reaggregated either immediately or after 4 hours. As observed before, dissociation for 4 hours followed by reaggregation led to expression of the pan-neural marker NCAM and suppression of epidermal keratin (EK; Jonas et al., 1985; Fig. 7B); addition of BMP4 at 50 ng/ml to the dissociated cells eliminated expression of the neural marker and restored EK expression. Injection of 2 ng of tXActRIIB led to neuralization even in the presence of BMP4 (Fig. 7B, lane 9). In contrast, injection of 2 ng of tXALK4 RNA did not change cell response to BMP4: NCAM was repressed and EK induced in BMP4-treated samples from both uninjected and tXALK4-injected embryos. In controls carried out as part of the same experiment, tXALK4 prevented mesoderm induction in intact caps by both activin and BMP4, confirming that the RNA was functional (data not shown). Thus, although tXALK4 blocks induction of mesoderm by BMP4 (Fig. 3), it fails to inhibit its epidermal induction activity.

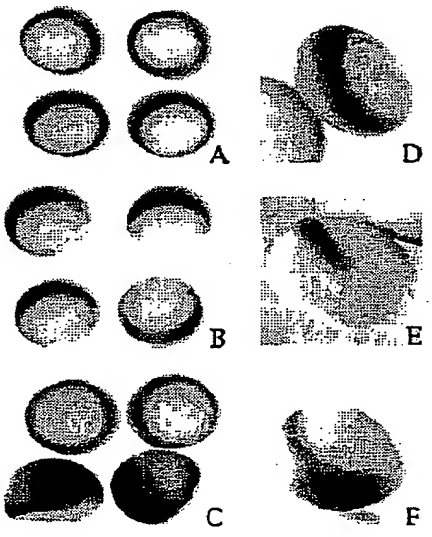


Fig. 5. Truncated XALK4 blocks mesoderm formation in vivo. Albino embryos were injected with 2 ng of tXALK4 or constitutively active ALK4-T206E RNAs in the marginal zone of one blastomere at the 2-cell stage. The embryos were fixed at gastrula stage 10 and whole-mount in situ hybridization was performed using an Xbra antisense probe to assess the effect of these constructs on mesoderm formation in vivo. (A,D) Uninjected control embryos, showing an intact ring of Xbra expression during early gastrula stages. (B,E) Embryos injected with tXALK4 RNA. Consistent with tXALK4 inhibition of mesoderm induction, Xbra expression is only detected in half of the marginal zone. (C,F) Embryos injected with ALK4-T206E RNA. These embryos display an expanded Xbra expression that includes the marginal zone and the animal cap on one side of the embryo.

DISCUSSION

In this paper, we report the characterization of a *Xenopus* type I activin receptor, XALK4, and an analysis of its function in early frog development. We find that, as in the case of the previously described type II activin receptor XActRIIB, a truncated form of XALK4 can block mesoderm induction by activin or related factors. Furthermore, an active form of ALK4 mimics activin-induced mesoderm formation. These results are consistent with cell culture data implicating this protein as a receptor that mediates activin effects. Unlike tXActRIIB, however, truncated XALK4 does not induce neural tissue. This paradox is explained by our finding that truncated XALK4 can block BMP4-induced expression of mesodermal markers, but not BMP4-induced expression of epidermal markers and inhibition of neuralization. In contrast, the dominant negative form of XActRIIB blocks both activities of BMP4. Thus the type I and type II activin receptors are involved in overlapping but distinct sets of embryonic signaling events.

XALK4 and mesoderm induction

Truncated XALK4 blocks mesoderm induction by activin and BMP4 in animal cap explants, as has been shown for the

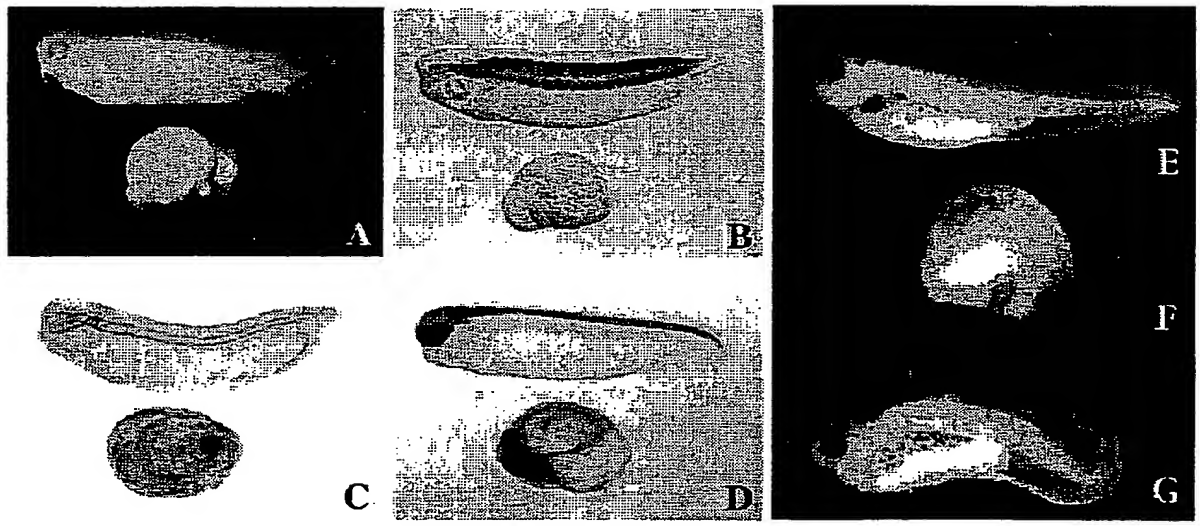
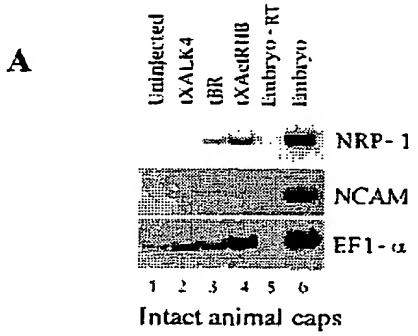


Fig. 6. Expression of tXALK4 in embryos leads to the elimination of the body axis and a severe reduction of mesodermal tissue. (A-D) Embryos at the top of each panels are uninjected controls, while the embryos at the bottom of each panels are injected with tXALK4. (A) Phenotype of embryos injected with 1 ng of tXALK4 RNA in the marginal zone of all four blastomeres at the 4-cell stage, display no obvious axis and resemble the 'bubble' phenotype described for tXActRIIB. (B-D) Analysis of tissue-specific molecular markers by whole-mount immunohistochemistry. (B) Staining with a muscle-specific antibody shows reduced muscle tissue in injected embryo. (C) Staining for a notochord antigen shows the same reduction in the injected embryo as above. (D) Staining for a neural-specific antigen demonstrates that neural tissue, though not reduced as severely, is disorganized. (E-G) The phenotype imposed by tXALK4 in the embryo can be rescued by coinjection of wild-type activin type 1 receptor. (E) Control uninjected tadpole. (F) Phenotype of embryo injected with 2 ng of tXALK4 into the two dorsal blastomeres in the marginal zone. (G) Coinjection of tXALK4 with 100 pg of the wild-type XALK4 RNA can rescue the phenotype and restore body axis in embryos.

truncated type II activin receptor tXActRIIB (Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou and Thomsen, 1995). Expression of the truncated form of either receptor strongly inhibits mesoderm formation in whole embryos as well, resulting in severely defective embryos which lack both heads and tails. Analysis of molecular markers reveals that expression of early genes, such as *Xbra*, is completely inhibited while development of late mesodermal tissues, such as muscle and notochord, is substantially reduced in these embryos. Moreover, a constitutively active form of ALK4 can induce mesoderm in *Xenopus* animal caps in a ligand-independent manner. These results strongly suggest that XALK4 plays an essential role in endogenous mesoderm induction, perhaps in conjunction with the type II receptor XActRIIB.



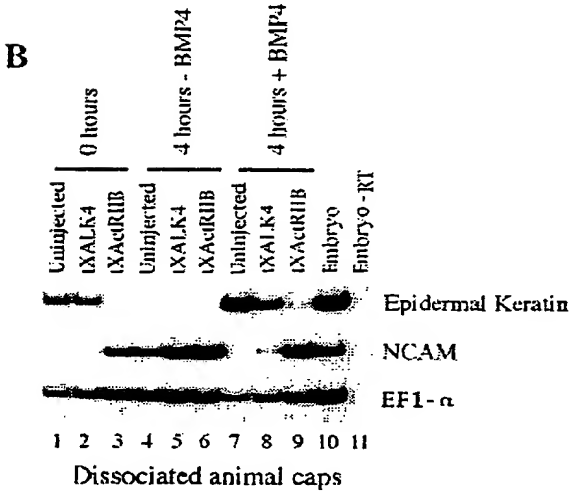


Fig. 7. tXALK4 does not induce neuralization in intact explants and does not inhibit epidermal induction by BMP4. (A) Ectodermal explants derived from embryos injected with tXALK4, tXActRIIB or tBR RNAs were assayed by RT-PCR for the expression of ectodermal-specific markers when sibling stages reached late neurula (stage 20). NRP-1 and NCAM are neural-specific markers. Lane 1 is uninjected animal cap control, lanes 2 to 4 are caps injected with RNA encoding tXALK4, tBR and tXActRIIB, respectively. Lane 5 is a negative control without reverse transcriptase, and lane 6 is a positive control with RNA extracted from whole embryos. tXALK4 does not neuralize intact ectodermal explants. (B) Ectodermal explants derived from embryos injected with tXALK4 or tXActRIIB were dissociated in Ca^{2+}/Mg^{2+}-free medium. They were reaggregated either immediately (lanes 1 to 3) or following 4 hours dissociation (lanes 4 to 9). As shown previously (Wilson and Hemmati-Brivanlou, 1995), 4 hours of dissociation changed animal caps from epidermal to a neural fate (lanes 4-6). In lanes 7 to 9, BMP4 protein was added at 50 ng/ml concentration during dissociated culture. Unlike tXActRIIB, expression of tXALK cannot block BMP4-dependent epidermal induction. Lanes 1, 4 and 7 are uninjected controls; lanes 2, 5 and 8 are embryos injected with tXALK4 RNA; lanes 3, 6 and 9 are injected with tXActRIIB RNA. Total RNA was extracted from explants and assayed by RT-PCR when control siblings reached late neurula stages 17 to 18.

Mesoderm induction by BMP4 protein requires high levels of ligand, approaching 1 μg/ml, or about 40 nM. On the contrary, unpublished data demonstrate that BMP4/BMP7 heterodimers can induce mesoderm at much lower (picomolar) concentrations (A. Suzuki and N. Ueno, personal communication). It is thus unclear whether BMP4 acts alone or with another factor to induce mesoderm in the embryo, or acts instead to ventralize mesoderm induced by other factors such as activin. Since ventralization is a likely role for BMP4 in vivo, it would be interesting to know if this activity could be blocked by tXALK4. We are currently addressing this issue. In animal cap assays, truncated XALK4 blocks mesoderm induction by both activin and BMP4. However, coexpression of wild-type XALK4 with the truncated form does not rescue mesoderm induction by BMP4, although induction by activin is restored (Fig. 4). For this reason, we believe it is unlikely that ALK4 is directly involved in BMP4 induction of mesoderm. The truncated mutant may block BMP4 indirectly, through interaction with another receptor (see below). This notion is also supported by our data that type II activin receptor, when coexpressed with tXALK4, can rescue mesoderm induction by BMP4.

Neural specification and the induction of epidermis: XALK4 and BMP4

Although the type I and type II activin receptors behave very similarly in assays of mesoderm induction, they differ in one crucial aspect. While truncated XActRIIB provokes formation of neural tissue in animal cap explants in the absence of mesoderm, truncated XALK4 does not. Several recent studies suggest that BMP4, rather than activin, is likely to be responsible for neural inhibition and epidermal specification in vertebrate embryos (Wilson and Hemmati-Brivanlou, 1995; Sasai et al., 1995; Hawley et al., 1995; Xu et al., 1995), and suggest that truncated XActRIIB probably neuralizes by blocking BMP4 signaling. We have asked therefore if the difference between type I and type II activin receptors with respect to neuralization is due to a difference in the ability to block BMP4

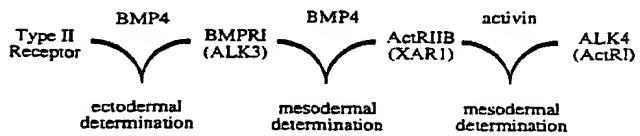


Fig. 8. Working model for differential involvement of XALK4 and XActRIIB in signal transduction by activin and BMP4. Different receptor complexes are involved in mesoderm and epidermis induction by activin and BMP4. For details, see Discussion.

signaling. We have shown that both truncated receptors can inhibit mesoderm induction by BMP4; however, in dissociated animal cap cells only tXActRIIB, and not tXALK4, can block epidermal induction by BMP4. Although the rescue data imply that XALK4 is not involved in either mesoderm or epidermis induction by BMP4, the differential effect of the truncated form of this receptor allows us to infer that different receptor complexes mediate the two activities.

To provide a framework to consider these data, we propose a model in which different ligands can induce distinct cellular responses through the formation of different receptor complexes (Fig. 8). Since we still know so little about the affinities of these receptors for each other and for the various ligands, and since there are a number of BMPs and receptors whose functions remain to be analyzed in the embryo, we limit this discussion to the molecules analyzed above. Under our current thinking, activin may signal through a receptor complex containing a type II receptor (ActRIIB) and a type I receptor (ALK4). A truncated form of either receptor subunit will block signaling and inhibit mesoderm induction by this ligand. On the other hand, BMP4 may mediate mesoderm induction by a BMPRI (ALK3) and ActRIIB complex, while specifying epidermal fate through BMPRI and another type II receptor (possibly a homologue of human BMPRII, Liu et al., 1995). In this case, a truncated BMPRI would block all BMP responses (Graff et al., 1994). A truncated ActRIIB would block BMP4-induced mesoderm formation directly and block epidermal formation indirectly by interacting with BMPRI and titrating it away from a still functional BMP type II receptor. A truncated ALK4, which would not complex with the type II BMP receptor, would not affect BMP4-mediated epidermal induction, although it would block BMP mesoderm induction by competing for ActRIIB. According to this model, coexpression of wild-type ALK4 should rescue mesoderm induction by activin in the presence of truncated ALK4 by restoring the functional receptor complex for activin signaling. However, BMP4-mediated mesoderm formation should not be rescued by expression of ALK4. This is exactly what we have observed (Fig. 4). In addition, mesoderm induction by BMP4 is rescued from tXALK4 by coexpression of XActRIIB, which further supports this model. Clearly the specific interactions will be determined by the relative affinities of the various components for each other. Therefore definitive conclusions must await further data, but we believe our model can serve as a useful starting point for design of experiments to probe the system in greater detail. A feature of the model is consistent with the possibility that a single type I receptor may mediate different intracellular events in combination with different type II receptors. Although a current hypothesis for receptor serine/threonine kinase signaling is that type I receptors are the primary signal transducers (Wrana et al., 1992, 1994; Attisano et al., 1996), a role for type II receptors in determining the nature of the downstream signal has also been proposed (Chen et al., 1993).

In summary, we have cloned a *Xenopus* type I activin receptor, XALK4, and studied its expression and function in early development. Using dominant negative and constitutively active mutants, we show that this receptor, like the type II receptor XActRIIB, is involved in mesoderm induction. In contrast, XALK4 is apparently not involved in the specification of epidermis and thus the control of neural fate. These experiments with dominant-negative receptors, together with the data on rescue with wild-type receptors, allow us to propose a model for how different receptor complexes mediate different biological activities of activin and BMP4.

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REFERENCES

Attisano, L., Wrana, J. L., Montalvo, E. and Massagué, J. (1996). Activation of signalling by the activin receptor complex. *Mol. Cell. Biol.* **16**, 1066-1073.

Boice, M. E., Hemmati-Brivanlou, A., Kushner, P. D. and Harland, R. M. (1992). Ventral ectoderm of *Xenopus* forms neural tissue, including hindbrain, in response to activin. *Development* **115**, 681-688.

Chen, R. H., Ebner, R. and Derynck, R. (1993). Inactivation of the type II receptor reveals two receptor pathways for the diverse TGF-beta activities. *Science* **260**, 1335-1338

Christian, J. L., Olson, D. J. and Moon, R. T. (1992). Xwnt-8 modifies the character of mesoderm induced by bFGF in isolated Xenopus ectoderm. *EMBO J.* **11**, 33-41

Dale, L., Howes, G., Price, B. M. J. and Smith, J. C. (1992). Bone Morphogenetic Protein 4: a ventralizing factor in Xenopus development. *Development* **115**, 573-585.

Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R. and Derynck, R. (1993). Cloning of a type I TGF-beta receptor and its effect on TGF-beta binding to the type II receptor. *Science* **260**, 1344-1348.

Fainsod, A., Steinbeisser, H. and De Robertis, E. M. (1994). On the function of BMP-4 in patterning the marginal zone of the Xenopus embryo. *EMBO J.* **13**, 5015-5025.

Gamer, L. W. and Wright, C. V. (1995). Autonomous endodermal determination in Xenopus: regulation of expression of the pancreatic gene XlHbox 8. *Dev. Biol.* **171**, 240-251.

Gilbert, S. F. (1992). *Developmental Biology*. Sunderland, MA: Sinauer.

Graff, J., Thies, R. S., Song, J. J., Celeste, A. J. and Melton, D. A. (1994). Studies with a Xenopus BMP receptor suggest that ventral mesoderm-inducing signals override dorsal signals in vivo. *Cell* **79**, 169-179.

Green, J. B. A., New, H. V. and Smith, J. C. (1992). Responses of embryonic Xenopus cells to activin and FGF are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* **71**, 731-739.

Green, J. B. A. and Smith, J. C. (1990). Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* **347**, 391-394.

Green, J. B. A. and Smith, J. C. (1991). Growth factors as morphogens: do gradients and thresholds establish body plan? *Trends In Genetics* **7**, 245-250.

Grunz, H. and Tacke, L. (1989). Neural differentiation of Xenopus laevis ectoderm takes place after disaggregation and delayed reaggregation without inducer. *Cell Diff. and Dev.* **28**, 211-218.

Gurdon, J. B., Harger, P., Mitchell, A. and Lemaire, P. (1994). Activin signalling and response to a morphogen gradient. *Nature* **371**, 487-492.

Harland, R. M. (1991). *In situ* hybridization: an improved wholemount method for *Xenopus* embryos. *Methods Cell Biology* **36**, 675-685.

Harland, R. M. (1994). The transforming growth factor beta family and induction of the vertebrate mesoderm: bone morphogenetic proteins are ventral inducers. *Proc. Natl. Acad. Sci. USA* **91**, 10255-10259.

Hawley, S. H., Wunnenberg-Stapleton, K., Hashimoto, C., Laurent, M. N., Watabe, T., Blumberg, B. W. and Cho, K. W. (1995). Disruption of BMP

signals in embryonic Xenopus ectoderm leads to direct neural induction. *Genes Dev.* **9**, 2923-2935.

Hemmati-Brivanlou, A., Frank, D., Bolce, M. E., Brown, R. D., Sive, H. L. and Harland, R. M. (1990). Localization of specific mRNAs in *Xenopus* embryos by whole-mount *in situ* hybridization. *Development* **110**, 325-330.

Hemmati-Brivanlou, A. and Harland, R. M. (1989). Expression of an *engrailed*-related protein is induced in the anterior neural ectoderm of early *Xenopus* embryos. *Development* **106**, 611-617.

Hemmati-Brivanlou, A., Wright, D. A. and Melton, D. A. (1992). Embryonic expression and functional analysis of a Xenopus activin receptor. *Developmental Dynamics* **194**, 1-11.

Hemmati-Brivanlou, A. and Melton, D. A. (1992). A truncated activin receptor inhibits mesoderm induction and formation of axial structures in Xenopus embryos. *Nature* **359**, 609-614.

Hemmati-Brivanlou, A. and Melton, D. A. (1994). Inhibition of activin receptor signaling promotes neuralization in Xenopus. *Cell* **77**, 273-281.

Hemmati-Brivanlou, A. and Thomsen, G. H. (1995). Ventral mesodermal patterning in Xenopus embryos: expression patterns and activities of BMP-2 and BMP-4. *Developmental Genetics* **17**, 78-89.

Henry, G. L., Brivanlou, I. H., Kessler, D. S., Hemmati-Brivanlou, A. and Melton, D. A. (1996). TGF-β signals and a pre-pattern in Xenopus laevis endodermal development. *Development* **122**, 1007-1015.

Jonas, E., Sargent, T. D. and Dawid, I. B. (1985). Epidermal keratin gene expressed in embryos of Xenopus laevis. *Proc. Natl. Acad. Sci. USA* **82**, 5413-5417.

Jones, C. M., Lyons, K. M., Lapan, P. M., Wright, C. V. E. and Hogan, B. J. M. (1992). DVR-4 (Bone Morphogenetic Protein-4) as a postero-ventralizing factor in Xenopus mesoderm induction. *Development* **115**, 639-647.

Kessler, D. S. and Melton, D. A. (1994). Vertebrate embryonic induction: mesoderm and neural patterning. *Science* **266**, 596-604.

Kessler, D. S. and Melton, D. A. (1995). Induction of dorsal mesoderm by soluble, mature Vg1 protein. *Development* **121**, 2155-2164.

Kintner, C. R. and Brockes, J. P. (1984). Monoclonal antibodies identify blastemal cells derived from dedifferentiating muscle in newt limb regeneration. *Nature* **308**, 67-69.

Kintner, C. R. and Melton, D. A. (1987). Expression of Xenopus N-CAM RNA in ectoderm is an early response to neural induction. *Development* **99**, 311-325.

Klein, P. S. and Melton, D. A. (1994). Hormonal regulation of embryogenesis: The formation of mesoderm in Xenopus laevis. *Endocrine Reviews* **15**, 326-341.

Koster, M., Plessow, S., Clement, J. H., Lorenz, A., Tiedemann, H. and Knochel, W. (1991). Bone Morphogenetic Protein 4 (BMP4), a member of the TGF-β family, in early embryos of *Xenopus laevis*: analysis of mesoderm inducing activity. *Mech. Dev.* **33**, 191-200.

Krieg, P. A. and Melton, D. A. (1984). Functional messenger RNAs are produced by SP6 in vitro transcription of cloned cDNAs. *Nucl. Acids Res.* **12**, 7057-7070.

Lamb, T. M. and Harland, R. M. (1995). Fibroblast growth factor is a direct neural inducer, which combined with noggin generates anterior-posterior neural pattern. *Development* **121**, 3527-3636.

Lin, H. Y., Wang, X.-F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H. F. (1992). Expression cloning of the TGF-β type II receptor, a functional transmembrane serine/threonine kinase. *Cell* **68**, 1-20.

Liu, F., Ventura, F., Doody, J. and Massagué, J. (1995). Human type II receptor for bone morphohenic proteins (BMPs): extension of the two-kinase receptor model to the BMPs. *Mol. Cell. Biol.* **15**, 3479-3486.

Mathews, L. S. and Vale, W. W. (1991). Expression cloning of an activin receptor, a predicted transmembrane serine kinase. *Cell* **65**, 973-982.

Mathews, L. S., Vale, W. W. and Kintner, C. R. (1992). Cloning of a second type of activin receptor and functional characterization in Xenopus embryos. *Science* **255**, 1702-1705.

Mathews, L. S. (1994). Activin receptors and cellular signaling by the receptor serine kinase family. *Endocrine Reviews* **15**, 310-325.

Nieuwkoop, P. D. and Faber, J. (1967). *Normal Table of Xenopus laevis (Daudin)*. Amsterdam: North Holland Publishing Company.

Richter, K., Good, P. J. and Dawid, I. B. (1990). A developmentally regulated, nervous system-specific gene in Xenopus encodes a putative RNA-binding protein. *New Biol.* **2**, 556-565.

Sanger, S., Nicklen, S. and Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**, 5463-5467.

Sasai, Y., Lu, B., Steinbeisser, H. and De Robertis, E. M. (1995). Regulation of neural induction by the Chd and BMP-4 antagonistic patterning signals in Xenopus. *Nature* **376**, 333-336.

Schmidt, J. E., Suzuki, A., Ueno, N. and Kimelman, D. (1995). Localized BMP-4 mediates dorsal/ventral patterning in the early Xenopus embryo. *Dev. Biol.* **169**, 37-50

Schulte-Merker, S., Smith, J. C. and Dale, L. (1994). Effects of truncated activin and FGF receptorsand of follistatin on the inducing activities of BVg1 and activin: does activin play a role in mesoderm induction? *EMBO J.* **13**, 3533-3541

Smith, J. C., Price, B. M. J., Green, J. B. A., Weigel, D. and Herrmann, B. G. (1991). Expression of a Xenopus homolog of Brachyury (T) is an immediate-early response to mesoderm induction. *Cell* **67**, 79-87.

Suzuki, A., Theis, R. S., Yamaji, N., Song, J. J., Wozney, J., Murakami, K. and Ueno, N. (1994). A truncated BMP receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc. Natl. Acad. Sci. USA* **91**, 10255-1259.

Symes, K., Yordan, C. and Mercola, M. (1994). Morphological differences in Xenopus embryonic mesodermal cells are specified as an early response to distinct threshold concentrations of activin. *Development* **120**, 2339-2346.

ten Dijke, P., Ichijo, H., Franzen, P., Schulz, P., Saras, J., Toyoshima, H., Heldin, C. H. and Miyazono, K. (1993). Activin receptor-like kinases: a novel subclass of cell-surface receptors with predicted serine/threonine kinase activity. *Oncogene* **8**, 2879-2887.

ten Dijke, P., Yamashita, H., Ichijo, H., Franzen, P., Laiho, M., Miyazono, K. and Heldin, C. H. (1994a). Characterization of type I receptors for transforming growth factor-beta and activin. *Science* **264**, 101-104.

ten Dijke, P., Yamashita, H., Sampath, T. K., Reddi, A. H., Estevez, M., Riddle, D. L., Ichijo, H., Heldin, C. H. and Miyazono, K. (1994b). Identification of type I receptors for osteogenic protein-1 and bone morphogenetic protein-4. *J. Biol. Chem.* **269**, 16985-16988.

Thomsen, G. H. and Melton, D. A. (1993). Processed Vg1 protein is an axial mesoderm inducer in Xenopus. *Cell* **74**, 433-441.

Weeks, D. L. and Melton, D. A. (1987). A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-β. *Cell* **51**, 861-867.

Willis, S. A., Zimmerman, C. L., Li, L. and Mathews, L. S. (1996). Formation and activation by phosphorylation of activin receptor complexes. *Molecular Endocrinology* **10**, 367-379.

Wilson, P. A. and Melton, D. A. (1994). Mesodermal patterning by an inducer gradient depends on secondary cell-cell communication. *Current Biology* **4**, 676-686.

Wilson, P. A. and Hemmati-Brivanlou, A. (1995). Induction of epidermis and inhibition of neural fate by Bmp-4. *Nature* **376**, 331-333.

Wrana, J. L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.-F. and Massagué, J. (1992). TGFβ signals through a heteromeric protein kinase receptor complex. *Cell* **71**, 1003-1014.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. and Massagué, J. (1994). Mechanism of activation of the TGF-beta receptor. *Nature* **370**, 341-347.

Xu, R.-H., Kim, J., Taira, M., Zhan, S., Sredni, D. and Kung, H.-F. (1995). A dorminant negative done morphogenetic protein 4 receptor causes neuralization in Xenopus ectoderm. *Biochem. Biophys. Res. Commun.* **212**, 212-219.

Yamashita, H., ten Dijke, P., Huylebroeck, D., Sampath, T. K., Andries, M., Smith, J. C., Heldin, C. H. and Miyazono, K. (1995). Osteogenic protein-1 binds to activin type II receptors and induces certain activin-like effects. *J. Cell Biol.* **130**, 217-226.

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